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# Barley Aleurone Xylanase: Purification, Characterization, Synthesis and Roles in Cell Wall Degradation and Enzyme Release

by

Em-on Benjavongkulchai

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Barley Aleurone Xylanase: Purification, Characterization, Synthesis and Roles in Cell Wall Degradation and Enzyme Release submitted by Em-on Benjavongkulchai in partial fulfilment of the requirements for the degree of Ph.D in Plant Biochemistry.

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Supervisor

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Date.....

*To my parents*

## Abstract

Xylanase from aleurone layers of Himalaya barley was purified by preparative isoelectric focusing and a Sephadex G-200 column. The molecular weight of the enzyme was found to be 34 or 27 kilodalton determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis or Bio-Gel P-200 chromatography, respectively. The isoelectric point was 4.6. The enzyme had maximum activity at pH 5.5 and at 35°C. It was most stable at pH 5-6 and at 0-4°C. The Michaelis-Menten constant was 0.86 mg of xylan/ml. The effects of some ions and inhibitors on the activity were also studied.

The activities of xylanase in aleurone layers and α-amylase in the incubation medium develop simultaneously during the first 24 h incubation with gibberellic acid. The release of α-amylase into the medium is detectable at 6 h. In the presence of gibberellic acid, higher levels of xylanase are induced. From 2 to 6% of the aleurone cell wall is hydrolyzed by xylanase after 6 h incubation, probably sufficient to permit the release of α-amylase. Scanning electron microscopy showed that purified xylanase hydrolyzed the aleurone cell walls in the absence of gibberellic acid. These observations suggest that xylanase plays an important role in the release of hydrolytic enzymes from the aleurone cells.

The production of xylanase in response to gibberellic acid was inhibited by cycloheximide and by cordycepin. Studies by density labeling of barley aleurone layers in the presence of gibberellic acid with $D_2O$ or with $H_2{}^{18}O$, and isopycnic equilibrium sedimentation were performed. Density shifts of 1.1 and 0.9% were obtained in the presence of $D_2O$ and $H_2{}^{18}O$, respectively. The results indicate that xylanase was synthesized *de novo* in response to gibberellic acid.

The synthesis of xylanase was dependent on both gibberellic acid and $Ca^{2+}$. Strontium ions and $Mg^{2+}$ could replace $Ca^{2+}$ for xylanase synthesis. The concentrations of $Ca^{2+}$ that gave maximum xylanase induction were 5-40 mM. Calmodulin did not improve xylanase activity from that obtained in the presence of gibberellic acid and $Ca^{2+}$; however, it minimized the inhibition of xylanase synthesis and release by chlorpromazine and trifluoperazine.

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## List of Abbreviations

| | |
|---|---|
| ABA | cis-abscisic acid |
| ADP | adenosine 5'-diphosphate |
| ATP | adenosine 5'-triphosphate |
| CaM | calmodulin |
| cAMP | adenosine 2',3'-cyclic monophosphate |
| CM-Sephadex | carboxymethyl Sephadex |
| CMC | carboxymethyl cellulose |
| CPZ | chlorpromazine |
| DEAE-cellulose | diethylaminoethyl cellulose |
| DEAE-Sephadex | diethylaminoethyl Sephadex |
| DNA | deoxyribonucleic acid |
| EGTA | ethylene glycol-bis($\beta$-aminoethyl ether)-N,N,N',N'-tetraacetic acid |
| ER | endoplasmic reticulum |
| GA | gibberellic acid |
| $GA_3$ | gibberellic acid $A_3$ |
| ID | inner diameter |
| kD | kilodalton |
| Km | Michaelis-Menten constant |
| mA | milliampere |
| $\mu$Ci | microcurie |
| mRNA | messenger ribonucleic acid |
| munits | milliunits |
| NADH | nicotinamide adenine dinucleotide (reduced form) |
| NADPH | nicotinamide adenine dinucleotide phosphate (reduced form) |
| PAGE | polyacrylamide gel electrophoresis |

| | |
|---|---|
| PAS | periodic acid-Schiff |
| pI | isoelectric point |
| PIEF | preparative isoelectric focusing |
| poly(A) | polyadenylic acid |
| RNA | ribonucleic acid |
| SD | standard deviation |
| SDS | sodium dodecyl sulfate |
| TFP | trifluoperazine |
| Tris | 2-amino-2-(hydroxymethyl)-1,3-propanediol |
| Vh | volt-hour |
| Vmax | maximum velocity |
| X | xylanase |

# I. INTRODUCTION

One of the most important events during seed germination is the supply of nutrients to the growing embryo. For monocotyledonous seeds, such as barley, the endosperm is the nutrient storage site. The mobilization of the nutrients is controlled by hormones called gibberellin or gibberellic acids (GA), which are produced by the embryonic axis and transported to the aleurone layer (Jones 1973a). Gibberellic acid induces the *de novo* synthesis of many hydrolytic enzymes, including α-amylase (Filner and Varner 1967) and protease (Jacobsen and Varner 1967) within cells of the aleurone layer. Gibberellic acid also induces the release of these enzymes and other proteins from the aleurone layer into the endosperm.

The release of these hydrolases is of interest. It has been shown that their release is accompanied by degradation of the aleurone cell wall (Ashford and Jacobsen 1974, Jones 1969c, Jones and Price 1970). In addition, it has been demonstrated that the release of α-amylase through the aleurone cell wall is diffusion-limited (Varner and Mense 1972), and the release of acid phosphatase through the cell wall occurs when the cell wall is degraded (Ashford and Jacobsen 1974). Since the principal polysaccharide of the aleurone cell wall is arabinoxylan (McNiel *et al.* 1975), xylanase, which hydrolyzes the xylan backbone of this compound, may play an important role in the aleurone cell wall hydrolysis. However, very little work has been done on barley aleurone xylanase. In fact, xylanase from higher plants has never been purified and characterized. In contrast to plant xylanase, xylanases from plant pathogens (e.g. fungi and bacteria) have been extentively studied (see review by Dekker and Richards 1976).

The intent of this research was to isolate, purify and characterize xylanase from barley aleurone cells and investigate its role with respect to the degradation of the aleurone cell wall and the release of hydrolytic enzymes (e.g. α-amylase) and ions (e.g. $PO_4^{3-}$). The synthesis and release of xylanase in response to $GA_3$ (the most commonly used GA in this field) and the influence of factors such as $Ca^{2+}$ and calmodulin, which are reported to play a role in the synthesis and release of barley aleurone α-amylase (Jones and Jacobsen 1983), are also

studied.

## II. LITERATURE REVIEW

### A. Xylanase: occurrence and action

Xylanases are enzymes that hydrolyze D-xylans to D-oligoxyloses or D-xylose and D-oligoxyloses and they belong to a group called hemicellulases. There are two types of xylanases, namely, $\beta$-1,3-D-xylanases (E.C. 3.2.1.72 and E.C. 3.2.1.32 for exo- and endoxylanases, respectively) that hydrolyze $\beta$-1,3 linkages of D-xylan, and $\beta$-1,4-D-xylanases (E.C. 3.2.1.37 and E.C. 3.2.1.8 for exo- and endoxylanases, respectively) that hydrolyze $\beta$-1,4 linkages of D-xylan (International union of biochemists 1979). The $\beta$-1,3-xylanases are rare and found only in microorganisms. Knowledge of this type of xylanase is limited. The $\beta$-1,4-xylanases have been detected in many microorganisms, invertebrates, marine algae and higher plant seeds, including those of wheat, maize, rye and barley (Dekker and Richards 1976, Preece and McDougall 1958). The term "xylanases" in the rest of this thesis refers to the $\beta$-1,4-group.

There are two major types of xylanase, based on the modes of action on the substrate xylan, namely, exo- and endoxylanases. Hydrolysis by exoxylanase starts from the terminal linkages of the substrate and its products are xylose and oligoxyloses, while hydrolysis by endoxylanase starts in the middle linkages of the substrate and its products are oligoxyloses. Barley aleurone layers produce both exo- and endoxylanases but endoxylanase appears to be the major type produced in response to gibberellic acid (Dashek and Chrispeels 1977, Eastwell 1981).

### B. Sequence of events during seed germination

The classical hypothesis of the events that take place during cereal seed germination is illustrated in Figure 1. The embryo produces a plant growth regulator, GA, which diffuses from the embryo into aleurone cells that surround the endosperm. In response to GA, the aleurone cells synthesize a number of hydrolases that are then released into the endosperm

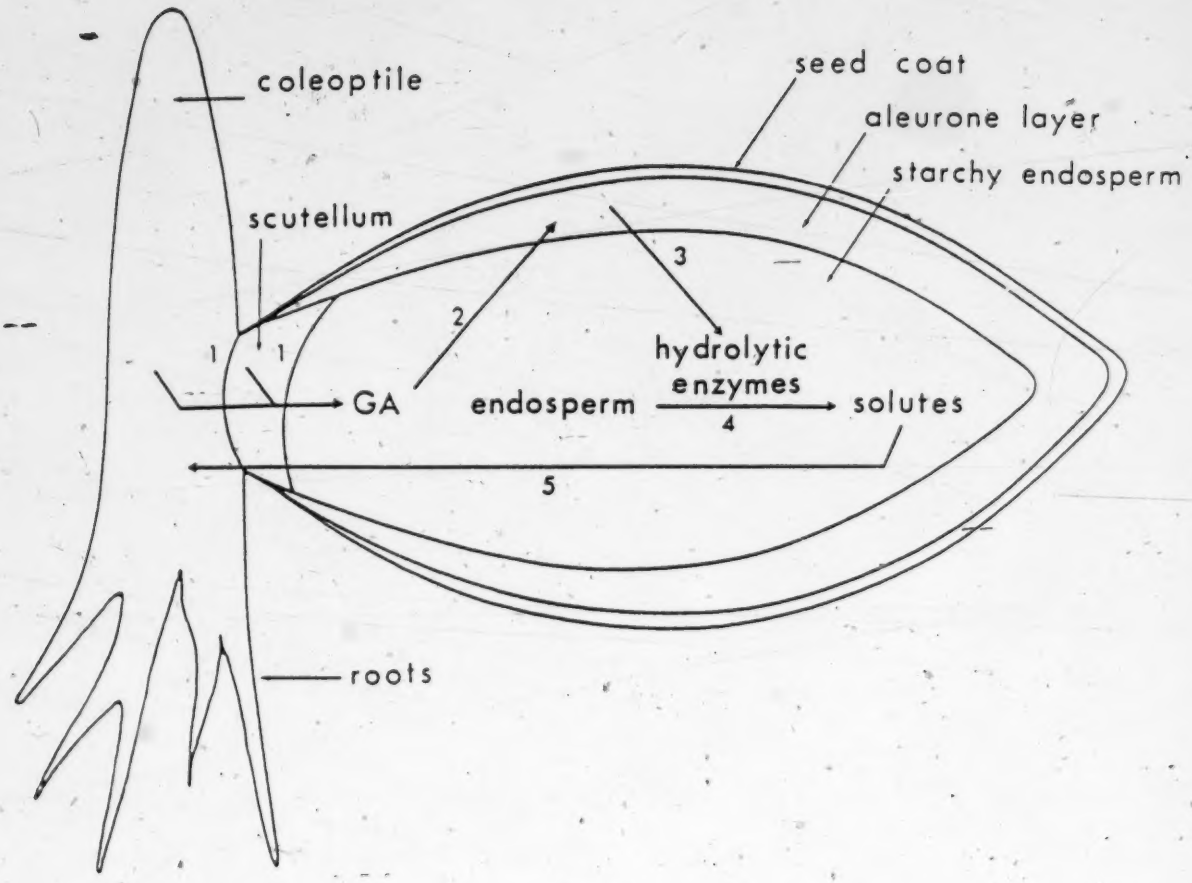



**Figure 1.** Diagrammatic representation of the relationships among production of gibberellic acid (GA), hydrolytic enzymes and solutes in germinating barley seeds. GA produced by the coleoptile and scutellum (1) migrates into aleurone layer (2) and induces the aleurone cells to synthesize hydrolytic enzymes that are then released into the endosperm (3). These enzymes hydrolyze the reserves in the endosperm (4), producing solutes to nourish the growing embryo (5). Modified from Jones and Armstrong 1971.

where they hydrolyze their polymeric substrates in the nutrient reserves into simple compounds. These hydrolytic products are then transported back to the embryo and used for its growth. This classical hypothesis is generally accepted and is supported by a number of lines of evidence including the discovery of GA in the embryo, as reviewed by Jones (1973a). The major GAs that have been reported to be present in the dry seed and/or germinating barley are conjugates of $GA_1$, $GA_3$ and $GA_4$ (Atzorn and Weiler 1983, Groat and Briggs 1969, Radley 1967). However, it is now controversial as to whether the conjugates of GA produced in the embryo take part in the enzyme induction in aleurone cells. Atzorn and Weiler (1983) found that $GA_4$ is produced by aleurone layers while Gilmour and MacMillan (1984) could not detect any $GA_4$ in germinating barley seeds. These topics have been recently reviewed (Akazawa and Hara-Nishimura 1985, Atzorn and Weiler 1983, Trewavas 1982).

**C. Composition and structure of aleurone cell walls**

The aleurone layer of barley seed consists of living, non-dividing and non-differentiating cells. The layer is 3-4 cells thick. The aleurone cells have unique characteristics. They have no vacuole, but thick cell walls, aleurone grains (protein and phytin storage), and spherosomes (lipid storage, Jones 1969a). Barley and wheat aleurone cell walls are distinctly bilayered (thick outer and thin inner wall layers), with a middle lamella (Bacic and Stone 1981a, Jones 1969c). An abundance of plasmodesmata is also observed (Bacic and Stone 1981a, Taiz and Jones 1973). Aleurone cell walls of barley are composed of two major polysaccharides, arabinoxylan and 1,3;1,4-$\beta$-glucan (Bacic and Stone 1981b, McNiel *et al.* 1975). Arabinoxylan, which is about 85% of total wall composition, has a $\beta$-1,4-xylan backbone, with 33% of the xylosyl residues substituted at the 2 and/or 3 position with single arabinofuranosyl groups (McNiel *et al.* 1975). Cellulose, protein (with no detectable hydroxyproline like wall proteins from other monocots) and ferulic acid (a phenolic compound) have been detected in the walls as well (Fincher 1976).

**D. Changes in aleurone cell structures during germination**

In the presence of $GA_3$, many changes in aleurone cells are observed during the first 24 h of germination. These changes involve proliferation of endoplasmic reticulum (ER), distention of ER cisternae, polysome formation and proliferation of vesicles from ER and dictyosomes (Evins and Varner 1972, Jones 1969c). The size of the aleurone grains, and the numbers of spherosomes and microbodies decrease while numbers of plastids increase and vacuoles begin to form (Jones 1969b, Jones 1969c, Jones and Price 1970). The development of mitochondrial cristae is enhanced during the imbibition. However, a crystalline inclusion develops in the mitochondria 24 h after $GA_3$ treatment and this may be related to the degeneration and subsequent reduced oxygen consumption of the aleurone cells (Jones and Price 1970).

Degradation of the aleurone cell wall is also observed (Ashford and Jacobsen 1974, Pomeranz 1972). Secondary (inner) walls are degraded after 20-22 h of exposure to $GA_3$ (Jones 1969c). However, the innermost part of the wall region, which, based on staining properties, has a different but unknown chemical composition from the rest of the wall, is resistant to hydrolysis (Ashford and Jacobsen 1974, Taiz and Jones 1970, Taiz and Jones 1973). The synthesis of pentose-containing cell wall macromolecules decreases (Johnson and Chrispeels 1973), while the release of cell wall pentoses increases (Dashek and Chrispeels 1977).

**E. Biochemical and physiological changes during germination**

Dry seeds respire at a very low rate, but once they take up water, the seeds begin to germinate. Respiration by the embryo, the aleurone layer and other living tissues dramatically increases to provide the energy (ATP, NAD(P)H) for biosynthetic processes (Bewley and Black 1985).

Protein and RNA syntheses are much increased in both germinating barley and in isolated, $GA_3$-treated aleurone layers. Many hydrolytic enzymes are synthesized and/or

released in response to $GA_3$. These enzymes can be divided into two groups based on their responses to $GA_3$. The synthesis and release of the first group are $GA_3$-dependent. The enzymes in this group include $\alpha$-amylase and some proteases. The second group of enzymes requires $GA_3$ only for their release, but not for their synthesis. Examples of enzymes in this group are $\beta$-glucanase, acid phosphatase and ribonuclease.

Among the hydrolases produced by aleurone cells, $\alpha$-amylase is the most abundant protein (about 60% of total protein synthesized after 24 h incubation with $GA_3$) and the most extensively studied. It is synthesized *de novo* in response to $GA_3$ (Filner and Varner 1967). The $GA_3$-induced increase in $\alpha$-amylase synthesis results from the increase in the level of mRNA for $\alpha$-amylase. $\alpha$-Amylase in cereal seeds has been reviewed by Ho (1979), Ho (1980) and Ho (1985).

**F. Cell wall hydrolyzing enzymes and $GA_3$**

Based on the composition of aleurone cell walls, the enzymes that might be involved in the cell wall degradation of barley aleurone are $\beta$-1,4-xylanase, $\beta$-xylopyranosidase, $\alpha$-arabinofuranosidase, protease, $\beta$-glucanase and cellulase. However, $GA_3$ only enhances the activity of the first four of these enzymes in isolated aleurone layers (Jacobsen and Varner 1967, Taiz and Honigman 1976). The activity of $\beta$-glucanase increases prior to exposure to $GA_3$ (Bennett and Chrispeels 1972, Jones 1971, Stuart 1986) and no $GA_3$-stimulated cellulase is detected (Taiz and Honigman 1975). There appears to be no clear evidence for degradation of cell wall proteins, although a wall-bound protease has recently been reported in *Phaseolus vulgaris* leaves (Fry 1985, Van der Wilden *et al.* 1983).

Among the three $GA_3$-induced arabinoxylan-hydrolyzing enzymes, the total activity of xylanase is much higher than the activity of the other two enzymes (Taiz and Honigman 1976). It was found that endoxylanase, rather than exoxylanase, is responsible for the initial degradation of arabinoxylan *in vitro* (Dashek and Chrispeels 1977, Eastwell 1981).

**G. Control of nutrient mobilization during seed germination**

The mobilization of nutrients during germination is controlled by plant hormones, the major ones being $GA_3$, abscisic acid (ABA) and ethylene. The control is via the synthesis and/or release of particular hydrolytic enzymes and proteins. The studies on the action of plant hormones usually use the classical aleurone tissue as an experimental system since the tissue consists of a homogeneous cell type and is only a few cells in depth so a uniform response should be easily obtained (Eastwell 1981).

$GA_3$ and ABA have been reported to induce the synthesis of many proteins (Ho 1982, Mozer 1982). In aleurone cells, the $GA_3$-induced activity of $\alpha$-amylase and protease is inhibited by ABA, probably by protein inhibitors induced by ABA (Hammerton and Ho 1986, Higgins *et al.* 1982, Mozer 1980). Ethylene can prevent the inhibitory effect of ABA on $GA_3$-enhanced $\alpha$-amylase in aleurone layers (Jacobsen 1973, Tittle and Spencer 1986, Varty *et al.* 1983). It was found to increase the release of $\alpha$-amylase into the medium as well (Eastwell and Spencer 1982a, Jacobsen 1973, Jones 1968). In addition, ethylene enhances $GA_3$-induced xylanase activity in aleurone layers (Eastwell and Spencer 1982b).

Calcium has been demonstrated to enhance the secretion (Moll and Jones 1982, Varner and Mense 1972) as well as the synthesis (Jones and Carbonell 1984, Mitsui *et al.* 1984) of $\alpha$-amylase. Moreover, the secretion of other hydrolases, e.g. acid phosphatase, $\beta$-glucanase, carboxypeptidase, protease and ribonuclease is also $Ca^{2+}$-dependent (Hammerton and Ho 1986, Jones and Jacobsen 1983). Other factors that may regulate the production of hydrolases in the germinating seed are osmoticum resulting from hydrolytic products (Jones and Armstrong 1971), cAMP, ADP (Galsky and Lippincott 1969), helminthosporic acid, helminthosporol (Okuda *et al.* 1967) and $CO_2$ (Tittle and Spencer 1986).

## III. MATERIALS AND METHODS

### A. Materials

Barley seeds (*Hordeum vulgare* L.cv. Himalaya, 1979 harvest) were obtained from the Agronomy Department, Washington State University, Pullman, Washington, USA. The seeds were stored at 4°C in a sealed glass jar until used.

All chemicals used were either analytical or reagent grade from J.T. Baker (Canlab, Edmonton, Alta, Canada), Bio-Rad (Bio-Rad Laboratories, Richmond, CA, USA), Fisher (Fisher Scientific, Edmonton, Alta, Canada), LKB (LKB Produkter AB, Bromma, Sweden), NEN (New England Nuclear, Boston, MA, USA), Pharmacia (Pharmacia (Canada) Inc., Dorval, Que, Canada), Sigma (Sigma Chemical Co., St. Louis, MO, USA), and Whatman (Whatman Ltd., Maidstone, Kent, UK).

### B. Preparation of half-seeds

Healthy seeds at least 6-7-mm in length were selected for the experiments. Half-seeds of barley were prepared according to the procedure described by Chrispeels and Varner (1967). The tip (approximately 1 mm) of distal end of each seed was cut off and from that point a 3-mm length of the seed was cut. This 3-mm segment of the seed was freed from the embryo portion and is called a half-seed.

One hundred half-seeds were surface sterilized by soaking in 50 ml of 75% (v/v) ethyl alcohol for 2 min and rinsing twice in 100 ml of sterile distilled water. All steps in sterilization of half-seeds were performed on a UV-sterilized laminar flow bench (Edgegard Hood model EG 4252, Baker Co., Inc., Sanford, ME, USA). Approximately 100 ml of 0.1 or 1% (v/v) NaOCl was then added to the half-seeds and left for 20 min. The half-seeds were rinsed three times, 5 min each, with 100 ml of sterile distilled water. Sixty to one hundred sterilized half-seeds were placed in a 250-ml sterile culture flask containing 3 layers of Whatman No. 1 filter paper (7 cm diameter) with 15 ml of sterile distilled water. The flask was wrapped in

aluminum foil, incubated in a 25°C water bath and connected to the air supply as described by Eastwell and Spencer (1982a).

**C. Preparation of aleurone layers**

Aleurone layers were prepared from half-seeds that had been imbibed for 4 days as described above. If there was any sign of microbial contamination, the contents of the flask were discarded. The microbial contamination was checked on a nutrient agar plate (Fisher). Aleurone layers from half-seeds sterilized in 1% NaOCl were separated from the endosperm and seed coats. These seed-coat-free layers are called isolated aleurone layers. The layers from half-seeds disinfected in 0.1% NaOCl had attached seed coats and are called aleurone layers. The separated layers were then rinsed with sterile distilled water until they were free of any endosperm residue.

**D. Incubation of aleurone layers**

Generally, aleurone layers were incubated at 22 or 25°C in either culture tubes or culture flasks. The system used was either closed or flow-through (Eastwell and Spencer 1982a). If a closed system was used, a culture flask was used to assure that there was enough oxygen for respiration. No difference was found in the xylanase production in the two systems. Routinely, the incubation medium was: 10 $\mu$M $GA_3$ (Sigma), 20 mM $Ca(NO_3)_2$ or $CaCl_2$, and 20 mM succinic acid, adjusted to pH 5.5 at 25°C with NaOH. At the beginning of this study, $CaCl_2$ was used to induce xylanase synthesis but it was found later that $Cl^-$ can inhibit xylanase activity; therefore, $NO_3^-$ was used instead. (Chloride ions did not inhibit the synthesis of xylanase.) Any variations in the incubation medium used are stated for each experiment. The pH of buffers used throughout the thesis was 5.5 at 25°C unless stated otherwise. (Xylanase was found to be most stable and active at this pH.)

**E. Xylanase assay**

The procedure for determination of xylanase activity was modified from that described by Taiz and Honigman (1976). Larchwood xylan (Sigma) was purified by alkaline copper precipitation (Jermyn 1955). In early experiments, the substrate solution consisted of 0.075% (w/v) purified xylan in 20 mM potassium acetate buffer and 20 mM $CaCl_2$ (pH 5.5). For all subsequent work, 0.2% (w/v) purified xylan in 20 mM succinate buffer and 2 mM $Ca(NO_3)_2$ was used because it was found later that 0.2% xylan gave higher activity than 0.075% xylan. The suspension was boiled for 1 min to dissolve the xylan and allowed to cool to 30°C.

The assay was performed by mixing 1 ml of xylan solution and 0.4 ml of sample at 30°C for 60 min. The reducing groups formed were determined by the Nelson method (1944) with anhydrous D-xylose as a reference standard. The absorbance of the sample was measured at 520 nm in a double-beam spectrophotometer (Cary 219, Varian Assoc., Inc., Palo Alto, CA, USA). The control for the assay was a zero-time sample in which the substrate and terminating reagent, alkaline copper reagent of the Nelson method (1944), were added simultaneously. One unit of xylanase activity was defined as the amount of enzyme required to expose 1 μmol reducing terminals per min at 30°C.

**F. Protein determinations**

The protein concentrations were evaluated by the method of Bradford (1976) with bovine serum albumin (fraction V, Sigma) as a standard. Absorbance measurements at 280 nm were used to estimate protein concentration after gel chromatography.

**G. Xylanase purification**

*Enzyme preparation*

Two hundred isolated aleurone layers were incubated for 72 h at 25°C in 250-ml sterile culture flask that contained 40 ml of 20 mM succinic acid, 20 mM $CaCl_2$ and 10 $\mu$M $GA_3$ (pH 5.5). The flask was supplied with hydrocarbon-free air in a flow-through system (Eastwell and Spencer 1982a).

All subsequent purification steps were performed at 4°C. After 72 h incubation with $GA_3$, the enzyme was isolated from the incubation medium as follows. The medium was centrifuged at 20,800 g for 10 min to clarify it. The enzyme in the supernatant solution was concentrated by precipitation overnight with $(NH_4)_2SO_4$ at 15-85% saturation. The precipitate was collected by centrifugation at 20,800 g for 10 min and kept in saturated $(NH_4)_2SO_4$ until enough sample was obtained. The precipitate was separated from the saturated $(NH_4)_2SO_4$ by centrifugation, redissolved in 20 mM potassium acetate buffer and dialyzed against the same buffer for 24 h. The dialyzed solution was centrifuged and concentrated by a Centricon microconcentrator (Amicon Canada Ltd., Oakville, Ont. Canada). The concentrated sample was applied to a preparative isoelectric focusing gel with Sephadex G-200 SF as a support medium.

*Preparative isoelectric focusing (PIEF)*

The system of PIEF used here was essentially as described by Frey and Radola (1982). A suspension of Sephadex G-200 SF (Pharmacia) was prepared in deionised distilled water. The gel suspension was then mixed with 40% (w/v) ampholyte (Ampholine, LKB) to get 2% (w/v) concentration with pH 4 to 6, prepared by mixing 4 parts of pH 3.5-5 ampholyte with 1 part of pH 5-7 ampholyte. The gel, 3-mm-thick, was formed on a 1-mm-thick glass plate (14 x 20 cm).

The enzyme sample was applied as a streak on a gel surface with a 1-ml tuberculin syringe, needle No. 26G1/2. Pharmacia electrode strips (0.7 x 1.0 x 14.0 cm) were soaked with anolyte (0.025 M aspartic acid and 0.025 M glutamic acid) or catholyte (2 M ethylene

diamine with 0.025 M arginine and 0.025 M lysine). The strips were placed at both ends of the forming gel.

Focusing was performed with a FBE-3000 base unit with an isoelectric focusing lid (Pharmacia) and a Buchler 3-1500 power supply (Buchler Instruments, Fort Lee, NJ, USA). Cooling water (4°C) was provided by circulation through a cooling water bath (Messgeräte-Werk Lauda RC20/T2, Brinkman, FRG). The sample was focused at 8,850 Vh (500 V for 12 h and 950 V for 3 h).

After the focusing was terminated, the gel was sliced into 0.8-cm in lenghts with a fractionation grid, the slices were suspended in 5 ml of acetate buffer and gently agitated overnight to elute the enzyme. The gel suspension was then centrifuged at 20,800 g for 10 min and the supernatant solution was collected and assayed for xylanase activity and protein concentration. Fractions of highest specific activity were combined and concentrated by using an Amicon concentrator (Amicon Canada Ltd.). The concentrated sample was then loaded on a Sephadex G-200 column.

*Gel filtration chromatography*

A suspension of Sephadex G-200 (Pharmacia) was prepared according to the manufacturer's directions. The gel was packed in a column (30 cm long, 1 cm diameter) and equilibrated with 20 mM potassium acetate buffer (pH 5.5). Enzyme sample was loaded on the column and eluted with the acetate buffer at a flow rate of 3 ml/h. The eluent was collected in 2-ml fractions and assayed for protein concentration and xylanase activity. Fractions of highest specific activity were pooled together and checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), described below.

## H. Characterization of barley aleurone xylanase

*Molecular weight determinations*

a. Molecular weight determination of purified xylanase on SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 7.5-15% linear gradient of acrylamide (Bio-Rad) was prepared (Margolis and Kendrick 1968) in a slab gel (0.15 x 14 x 17.7 cm) and run in the discontinuous buffer system of Neville (1971) modified by Chua (1980). The linear gradient of acrylamide was formed by a gradient-maker (Buchler Instruments, Fort Lee, NJ, USA). Purified xylanase (10 $\mu$g) and the molecular weight markers were run at 16 mA/gel. The molecular weight markers were bovine serum albumin (66 kD), ovalbumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), trypsin inhibitor (20 kD), and $\alpha$-lactalbumin (14 kD). After electrophoresis, the gel was stained with Coomassie Blue R-250 (Sigma), destained in 7% glacial acetic acid and 40% methyl alcohol, and dried according to Wallevik *et al.* (1982).

b. Molecular weight determination of undenatured xylanase from the crude enzyme preparation, by Bio-Gel P-200 gel filtration chromatography

Bio-Gel P-200 (Bio-Rad) was prepared according to the manufacturer's directions, packed in a column (50 cm long, 0.9 cm diameter) and equilibrated with 20 mM succinic acid 2 mM in $Ca(NO_3)_2$ (pH 5.5). The crude xylanase was prepared by incubation of the aleurone layers with the routine incubation medium for 72 h at 25°C. After 72 h, the incubation medium was concentrated by an Amicon concentrator (Amicon Canada Ltd.) and loaded on the Bio-Gel P-200 column with a flow rate of 3 ml/h. Fractions (2 ml) were collected and assayed for xylanase activity and protein. The molecular weight markers (Sigma) or blue dextran (Pharmacia) were loaded separately on the same column. The molecular weight markers used were bovine serum albumin (66 kD), ovalbumin (45 kD), carbonic anhydrase (29 kD), myoglobin (17 kD) and horse heart cytochrome C (13 kD). The absorbance at 280

nm was used to locate the markers.

*Glycoprotein assay*

The purified xylanase on SDS-PAGE, prepared as described above, was stained for glycoprotein by use of the periodic-acid-Schiff (PAS) reaction (Zacharius and Zell 1969) with ovalbumin as a control.

*Isoelectric point determination*

Analytical isoelectric focusing of xylanase was performed in 12-cm long gels cast in glass cylinders (15 cm x 0.30 cm ID). The gel was: 5% (w/v) acrylamide, 0.13% (w/v) N,N'-methylene-bis-acrylamide, 0.1% (v/v) N,N,N',N'-tetramethyl-ethylene diamine, 0.05% (w/v) ammonium persulphate (Bio-Rad) and 2.5% (w/v) ampholyte (pH ranges 4 to 6 obtained as described in the "PIEF"). The anolyte was 40 mM aspartic acid and catholyte was 40 mM NaOH. The enzyme was applied and overlayed (3-5-mm in height) with an aqueous solution of 5% (w/v) glycerol and 0.5% (w/v) ampholyte, and focused at 3,900 Vh (150 V for 18 h, 600 V for 30 min and 900 V for 1 h) in a Bio-Rad 155 electrophoresis cell. After focusing, the gel was stained for protein by Coomassie Blue G-250 (Reisner *et al.* 1975). The enzyme activity was determined by cutting the gel into 0.5-cm lengths and assaying. For pH determination, the gel was cut into 0.5-cm lengths and shaken gently overnight with 2 ml of 10 mM KCl (Righetti and Drysdale 1976). The pH of the solution was read with a Microprocessor pH meter 811 (Orion Research Inc., Cambridge, MA, USA). The pH of the focused gel was also directly determined by use of a micro pH-electrode (Ingold Electrodes Inc., Andover, MA, USA).

**I. Kinetic studies**

*Rate of xylan hydrolysis*

Purified xylanase (0.24 $\mu$g) was assayed as described in the "Xylanase assay" at different times, i.e. 15 min intervals for 90 min.

*Effects of xylan concentration on activity*

The activity of purified xylanase was determined with various amounts (0.025 to 10 mg) of xylan prepared in acetate buffer 2 mM in $Ca(NO_3)_2$.

*Effects of pH on activity and stability*

The effect of pH on enzyme activity was studied by measuring xylanase activity, as described in the "Xylanase assay", except that the pH of the assay system was varied from 3.0 to 9.0. For pH values between 3.0 and 5.5, 20 mM citrate-HCl buffer was used; between 5.5 and 7.0, 20 mM sodium phosphate buffer; and between 8.0 and 9.0, 20 mM Tris-HCl buffer was used. The effect of pH on enzyme stability was studied by preincubation of purified xylanase with the appropriate buffer (pH 3.0 to 9.0) for 24 h at 4°C. After the preincubation, an aliquot was removed and assayed for activity.

*Effects of temperature on activity and stability*

The effect of temperature on enzyme activity was determined by assaying xylanase activity as described above except that the reaction mixture was incubated at different temperatures (4 to 60°C) for 30 min. For studies of the effect of temperature on enzyme stability, the purified enzyme solution was kept in acetate buffer, for 2 and 24 h at different temperatures (-12 to 40°C), and residual enzyme activity was measured.

*Effects of ions and inhibitors on activity*

Purified xylanase (4.26 x $10^{-3}$ units) was mixed with the following compounds, prepared in acetate buffer, to give two different final concentrations (2 and 20 mM). The tested compounds were $HgCl_2$, $Cu(NO_3)_2$, $Ca(NO_3)_2$, $CaCl_2$, $ZnCl_2$, NaCl, $NaNO_3$, $Na_2SO_4$, SDS, $KBrO_3$ and EGTA. The activity of xylanase was assayed as described in the "Xylanase assay".

**J. Role of xylanase in cell wall degradation and enzyme release**

*Time course study*

Aleurone layers (10 or 50) were incubated for various times at 22°C in darkness in medium composed of 20 mM succinate buffer and 20 mM $Ca(NO_3)_2$ in the presence or absence of 10 $\mu$M $GA_3$ in either closed system culture flasks or flow-through system culture tubes. The ratio of the number of layers to the volume (ml) of incubation medium was kept constant at 5 to 1. At selected times, the medium was removed and centrifuged at 20,800 g for 10 min. The supernatant solution was reduced to 1-2 ml using a Centricon microconcentrator. The layers were rinsed three times with succinate buffer with 2 mM $Ca(NO_3)_2$ and homogenized in 1 or 2 ml of the same buffer with a glass hand-homogenizer (Kontes Glass Co., Vineland, NJ, USA) and centrifuged. The layer extracts and concentrated media were divided into two portions. One of them was used for $K^+$, $Mg^{2+}$ and $PO_4^{3-}$ determinations. The other was dialyzed overnight against the succinate buffer and assayed for xylanase and $\alpha$-amylase activities.

Phosphate was determined according to the method of Serrano *et al.* (1976) except that 1% (w/v) ascorbic acid was used instead of 10%. One millilitre of the sample was mixed with 2 ml of 0.7% (w/v) ammonium molybdate in 0.72 N $H_2SO_4$ and 50 $\mu$l of 1% (w/v) ascorbic acid, left for 10 min and then the absorbance was read at 750 nm, with $K_2HPO_4$ as standard.

Concentrations of $K^+$ and $Mg^{2+}$ were determined by dilution of the samples to appropriate volume with 5% $HNO_3$, followed by measurement with a Perkin-Elmer 4000 atomic absorption spectrometer (Perkin-Elmer Corp., Norwalk, CT, USA). Potassium was measured in the emission mode at 766.5 nm and $Mg^{2+}$ in the absorption mode at 285.2 nm. Both ions were analyzed in an air-acetylene flame. The standard solutions for $K^+$ and $Mg^{2+}$ were $KNO_3$ and $MgSO_4$ respectively.

Determination of α-amylase was performed as described by Robyt and Whelan (1968) using the Nelson reagent (1944) with maltose as a standard. Fifty microlitres of sample was incubated with 1 ml of 1% starch solution (prepared in succinate buffer with 20 mM $CaCl_2$) at 30°C for 10 min. The reaction was terminated with 1 ml copper reagent and the reducing groups were determined as described above in the "Xylanase assay". One unit of α-amylase was defined as the amount of enzyme required to expose 1 μmol reducing terminals per min at 30 °C.

*Effects of purified xylanase on aleurone cell wall*

Twenty-five isolated aleurone layers were incubated at 25°C in 20 mM potassium acetate and 2 mM $Ca(NO_3)_2$ (pH 5.5) in the absence or presence of 0.10 units of purified xylanase (without $GA_3$) for 4, 20 and 50 h in a flow-through culture tube. After each time interval, five layers were removed and kept in 95% ethyl alcohol at -10°C. The layers were then frozen in liquid nitrogen, fractured and critical-point dried with $CO_2$. The layers were glued on stubs, gold coated and viewed on a Cambridge Stereoscan 250 scanning electron microscope (Cambridge Instruments Inc., Montreal, Que., Canada).

**K. $GA_3$ and xylanase synthesis**

*Effects of cycloheximide and cordycepin on xylanase synthesis*

Ten aleurone layers were incubated for 72 h in the routine incubation medium in the presence or absence of 10 μg/ml final concentration of cycloheximide or cordycepin (Sigma). In other experiments, the inhibitors were added after 12 h incubation with GA, and the incubation was continued for another 60 h. After the treatment, the medium and the layer extract were prepared as described in the "Time course study" and xylanase activity was assayed.

*De novo synthesis of xylanase*

Aleurone layers were incubated at 25°C for 120 h with the routine incubation medium prepared in $H_2O$, 75% $D_2O$ (99.8% isotopic purity, Merck Sharp & Dohme Isotope, Dorval, Que., Canada) or 80% $H_2{}^{18}O$ (97.4% isotopic purity, Merck Sharp & Dohme Isotope), respectively. (The ratio of the number of layers to the volume (ml) of incubation medium was 5 to 1.) After this period, the enzyme was prepared from the incubation medium and the layer extract as described in the "Time course study". The enzymes from both sources were combined, desalted and concentrated by a Centricon microconcentrator. The sample was then mixed with about 0.05 μCi of radioactive protein marker, (methyl-$^{14}$C) methylated ovalbumin (New England Nuclear, Boston, MA, USA), and saturated CsCl solution (density gradient grade, Beckman Instruments, Inc., Palo Alto, CA, USA) for an isopycnic equilibrium centrifugation as described by Filner and Varner (1967). The mixture was centrifuged at 40,000 rpm for 65 h in a Beckman L8-80 ultracentrifuge equipped with a SW 50.1 swinging bucket rotor (Beckman Instruments).

After centrifugation, the tubes were punctured at the bottom and one-drop fractions were collected. The alternate fractions were assayed for xylanase activity as described in the "Xylanase assay". The radioactivity in the alternate fractions was determined by adding 1 ml of distilled water to each fraction to dilute CsCl (Chrispeels and Varner 1973) and then 10 ml of Ready-solv™MP (Beckman instruments) or Aquasol 2 (NEN) and was counted by an

ISOCAP/300 liquid scintillation counter (Searle Analytic Inc., Des Plaines, IL, USA). The refractive index in about every eighth fraction was determined by an Abbe-refractometer (model A, Carl Zeiss, FRG). In some experiments, an aliquot of each fraction was assayed for the enzyme activity or radioactivity.

**L. Role of calcium and calmodulin on the synthesis and release of xylanase**

*Effects of calcium and other ions*

Ten aleurone layers were incubated in each of the following media:

1. 20 mM succinic acid buffer (pH 5.5)
2. 20 mM succinic acid buffer + 10 $\mu$M $GA_3$
3. 20 mM succinic acid buffer + 20 mM $Ca(NO_3)_2$
4. 20 mM succinic acid buffer + 10 $\mu$M $GA_3$ + 20 mM $Ca(NO_3)_2$
5. 20 mM succinic acid buffer + 10 $\mu$M $GA_3$ + 20 mM $CaCl_2$
6. 20 mM succinic acid buffer + 10 $\mu$M $GA_3$ + 40 mM $NaNO_3$
7. 20 mM succinic acid buffer + 10 $\mu$M $GA_3$ + 20 mM $Mg(NO_3)_2$
8. 20 mM succinic acid buffer + 10 $\mu$M $GA_3$ + 20 mM $SrCl_2$

The incubation was performed at 25°C for 72 h. After incubation, the medium and the layer extract were prepared as previously described in the "Time course study" and xylanase activity was assayed.

*Effects of calcium concentration*

Ten aleurone layers were incubated for 72 h at 25°C in 20 mM succinate buffer, 10 $\mu$M $GA_3$ and various concentrations (0-40 mM) of $Ca(NO_3)_2$. After incubation, the medium and the layer extract were prepared as previously described in the "Time course study" and xylanase activity was assayed.

*Effects of calmodulin and calmodulin antagonists*

Ten aleurone layers were incubated for 72 h in routine incubation medium in the presence or absence of one of the followings: 30 or 500 μM chlorpromazine (CPZ; Sigma), 30 or 500 μM trifluoperazine (TFP; Sigma), 0.07 or 5 μg/ml of calmodulin (CaM; bovine brain, Sigma), 30 μM CPZ + 5 μg/ml CaM, 500 μM CPZ + 0.07 μg/ml CaM, 30 μM TFP + 5 μg/ml CaM or 500 μM TFP + 0.07 μg/ml CaM. The medium and layer extract were prepared according to the "Time course study" and xylanase activity was assayed.

## IV. RESULTS AND DISCUSSION

### A. Sterilization of barley half-seeds

Most workers have sterilized the half-seeds in a high concentration (1-4% v/v) of NaOCl for 15-20 min (e.g. Chrispeels and Varner 1967, Eastwell and Spencer 1982a). However, recently Goudey *et al.* (1986) have shown that a high concentration (1% v/v) of NaOCl used for sterilization decreases the activity of barley aleurone α-amylase in response to $GA_3$ compared to a lower concentration (0.1% v/v) of NaOCl. Therefore, the effect of NaOCl on barley aleurone xylanase was investigated. The layers were prepared from the half-seeds sterilized in either 0.1 or 1% (v/v) NaOCl and incubated in the routine incubation medium. The results showed that the level of xylanase from layers sterilized in 0.1% NaOCl was higher than that from layers sterilized in 1% NaOCl (Table 1). The decrease in the enzyme levels in the presence of high concentration of NaOCl may result from the tissue damage caused by NaOCl (Goudey *et al.* 1986). After this discovery, 0.1% NaOCl was used for sterilization.

### B. Incubation system and dose response of $GA_3$ on xylanase levels

The incubation system used to induce xylanase in aleurone layers was that commonly used for α-amylase. This system has been reviewed and further investigated by Eastwell (1981). In the present study on xylanase, preliminary experiments showed that the optimal $GA_3$ concentration for the response of xylanase was 10 μM (results not shown), as also obtained by Eastwell (1981).

### C. Preliminary investigation of barley xylanase

The amount of xylanase present in aleurone layers is very low (Table 1), compared to that of α-amylase (Varner and Ho 1976); therefore, it was difficult to obtain enough enzyme for purification. In our preliminary study, it was shown that xylanase is only present in the

**Table 1.** Effects of different concentrations of NaOCl on the levels of barley aleurone xylanase. The half-seeds of barley were surface-sterilized and the aleurone layers were prepared as described in Materials and methods. Ten aleurone layers were incubated in 20 mM succinic acid, 10 μM GA$_3$ and 20 mM $Ca(NO_3)_2$, (pH 5.5) for 24, 48 and 72 h at 25°C. Xylanase in the incubation medium and in the layer extract was prepared and assayed as described in "Time course study" in Materials and methods. The values represent the mean of at least four different determinations ±SD.

| Incubation time (h) | Xylanase activity (munits/layer) | | | |
|---|---|---|---|---|
| | 0.1% NaOCl | | 1% NaOCl | |
| | Medium | Extract | Medium | Extract |
| 24 | 0.43 ± 0.25 | 3.52 ± 0.03 | 0.06 ± 0.01 | 1.45 ± 0.35 |
| 48 | 0.98 ± 0.01 | 3.57 ± 0.72 | 1.89 ± 0.26 | 0.94 ± 0.45 |
| 72 | 16.14 ± 4.18 | 0.89 ± 0.20 | 2.95 ± 0.36 | 0.02 ± 0.02 |

aleurone layer (results not shown). No xylanase activity was detected in the starchy endosperm, seed coat, embryo or scutellum. α-Amylase is present in both aleurone layers and scutellum (Akazawa and Miyata 1982). Half-seeds could be used as a starting material to avoid the time-consuming step in the separation of aleurone layers. Nevertheless, aleurone layers were used as the half-seeds had very high contents of interfering proteins. (Their protein content was about 5-6 times higher than that of aleurone layers.) Although the manual separation of aleurone layers is tedious and time-consuming, it is the best way to obtain aleurone layers with minimum damage to the tissue. An attempt to prepare aleurone tissues by use of a pearling machine was made but it was unsuccessful, possibly because of injury to the cell components caused by the vigorous action of the machine.

The low level of xylanase found in the aleurone layer preparation may result from binding of xylanase to the membrane or cell wall in the discarded pellet, or from hydrolysis of xylanase by protease. Experiments were performed to investigate these possibilities. The pellet was divided into two portions. The first portion was rinsed twice with 20 mM potassium acetate buffer (pH 5.5) and resuspended in 4 M LiCl or 4 M urea and agitated for 4 h to extract the enzyme from the cell walls. The extraction procedures were modified from the systems used for extracting α-mannosidase from oat coleoptile cell walls and autolytic enzymes from bacterial cell walls (Greve and Ordin 1977, Pooley *et al.* 1970). The second portion of the pellet was resuspended in acetate buffer and sonicated for 20 min in a Cole-Parmer sonic cleaner (model 30, Cole Parmer Instruments, Chicago, IL, USA) to release the enzyme from the membrane. This sonication was used to release α-amylase from the membrane of aleurone cells (Locy and Kende 1978, Tittle and Spencer 1986). After agitation or sonication, the suspension was centrifuged and the supernatant layer was dialyzed overnight against the acetate buffer.

There was no detectable xylanase activity in the supernatant layer from any of the treatments. Therefore, xylanase is not a membrane or cell wall bound enzyme and the total activity of xylanase could be accurately obtained from the medium and the extract. Although

LiCl and urea at 4 M were found to inhibit the activity of isolated xylanase completely, 60 and 80%, respectively, of the activity could be recovered by dialysis.

It is known that protease is present in the enzyme preparation from aleurone layers (Eastwell and Spencer 1982c, Hammerton and Ho 1986, Jacobsen and Varner 1967). However, addition of the protease inhibitor, phenylmethyl sulfonyl fluoride (1 mM final concentration), to the crude enzyme preparation did not change the total activity of xylanase. It should be noted that the crude enzyme preparation, after passing through a Bio-Gel P-200 (Bio-Rad) gel filtration column, gave two peaks of xylanase, named as $X_1$ and $X_2$ (Figure 2). The molecular size of $X_1$ was larger than that of $X_2$. Therefore $X_2$ may come from the degradation of $X_1$ possibly by protease action. The degradation occurred without any change in total activity. The crude enzyme preparation kept at 4°C gave a smaller peak of $X_2$ (Figure 2a) than that kept at 22°C (Figure 2b). Moreover, after a second Bio-Gel P-200 treatment, there seemed to be no degradation of $X_1$. When $X_1$ was rechromatographed on Bio-Gel P-200, there was only one peak at the $X_1$ position. The protease may have been removed by the first Bio-Gel P-200 column, or the action of protease became less effective possibly by lack of activators and/or instability of the protease enzyme itself.

**D. Multiple forms of barley aleurone xylanase obtained by ion-exchange chromatography**

The survey experiments (before the actual procedure for the enzyme purification was chosen) were performed by applying the crude enzyme preparation (i.e. enzyme in the incubation medium) to a DEAE-Sephadex A50 (Pharmacia) or a DEAE-cellulose (DE-52, Whatman) column equilibrated with 20 mM potassium acetate buffer and 20 mM $CaCl_2$ (pH 5.5). The column was washed at a flow rate of 6 ml/h with two-bed volumes of the same buffer, followed by two-bed volumes of a linear gradient of 0.02-0.5 M $CaCl_2$.

From the DEAE-Sephadex column (Figure 3), there were two peaks of xylanase. One of them came out in the wash fractions (Xa), which indicated that this Xa had a pI>5.5. The other (Xb) bound to the DEAE-Sephadex and was eluted by $CaCl_2$. The Xb, which had a

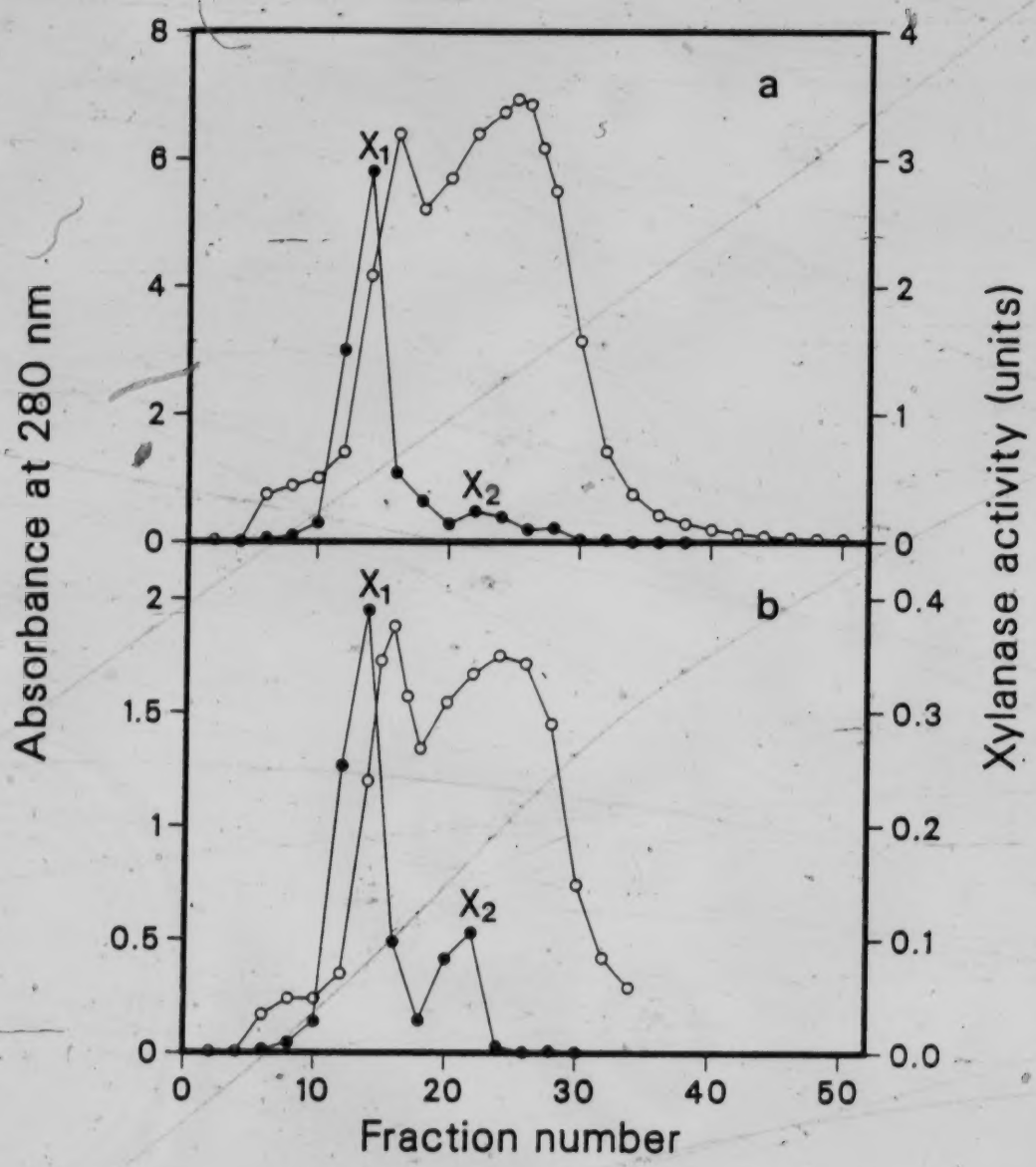



**Figure 2.** Gel filtration chromatography on Bio-Gel P-200 of the crude barley aleurone xylanase preparation kept at 4°C (a) or 22°C (b). The column (50 cm long, 0.9 cm diameter) was equilibrated and eluted with 20 mM succinic acid buffer (pH 5.5) 2 mM in $Ca(NO_3)_2$. Fractions (2.0 ml) were collected at a flow rate of 3 ml/h. •-• Xylanase activity ($X_1$, $X_2$), o-o absorbance at 280 nm.

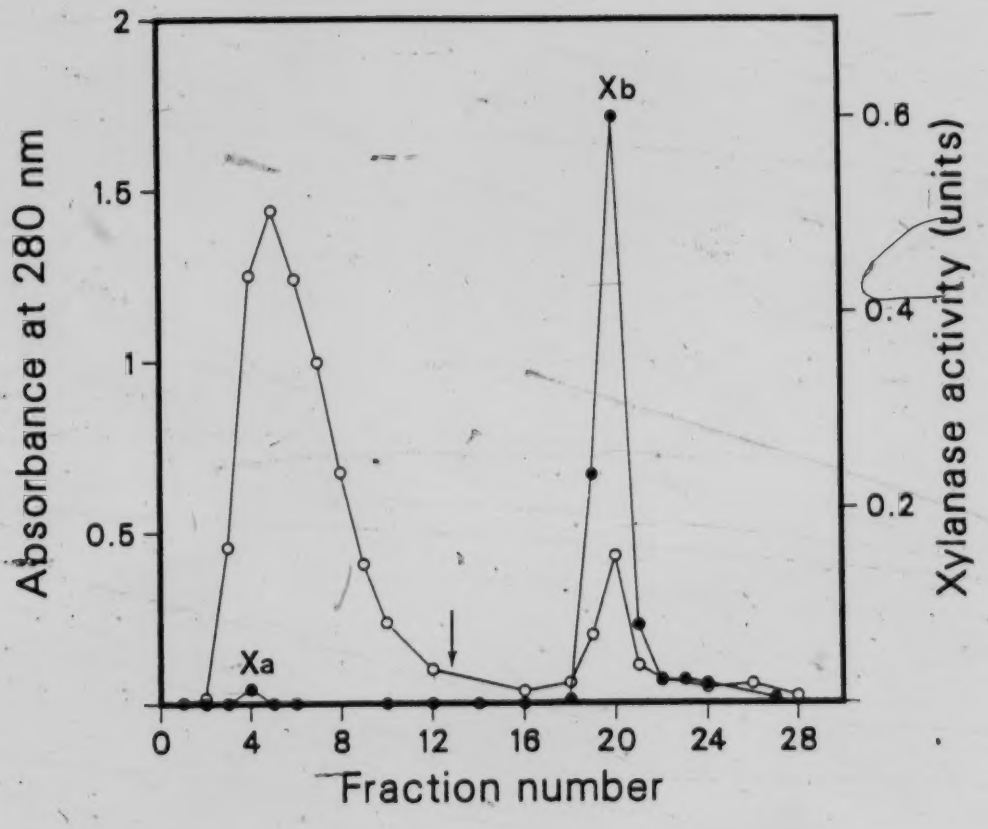



**Figure 3.** Ion exchange chromatography on DEAE-Sephadex A50 of the crude barley aleurone xylanase preparation from aleurone layers. The column (13 cm long, 1 cm diameter) was equilibrated with 20 mM potassium acetate buffer (pH 5.5) 20 mM in $CaCl_2$. A linear gradient of 0.02-0.5 M $CaCl_2$ was applied. Fractions (2.0 ml) were collected at a flow rate of 6 ml/h. •-• Xylanase activity (Xa, Xb), o-o absorbance at 280 nm. The arrow indicates the application of gradient.

pI<5.5, was at least 30-40 times as active as Xa. In some experiments, there was no detectable Xa. However, in the crude enzyme preparation from half-seeds that had been incubated with $GA_3$ in the absence of a 4-day imbibition period, the activity of Xa was higher than that of Xb (Figure 4). To be certain that Xa was really another form of xylanase, not an overloaded Xb, the Xa fraction was rechromatographed on CM-Sephadex C50 (Pharmacia) under the same conditions as for DEAE-Sephadex A50 except that a 0-0.5 M linear gradient of NaCl was used instead of the $CaCl_2$ gradient and $Ca^{2+}$ was omitted from the acetate buffer. (Calcium made the CM-Sephadex gel shrink and altered the appearance of the gel.) Under these conditions, Xa bound to CM-Sephadex and could be eluted by NaCl (Figure 5). This result clearly showed that Xa was one form of xylanase. It is interesting that the levels of Xa and Xb were different between aleurone layers and half-seeds (Figures 3 and 4). This may result from the changes in the requirement of isozymes for seed germination with time since the 4-day period of imbibition was omitted when the half-seeds were used. The multiplicity of barley $\alpha$-amylases as well as the change in isozyme pattern with time has also been shown (Bøg-Hansen and Daussant 1974, Jacobsen *et al.* 1970, Jacobsen and Higgins 1982, MacGregor and Daussant 1979). It was noted that when Xb was passed through the Bio-Gel P-200 column, it gave $X_1$ and $X_2$.

The results with DEAE-cellulose were surprising. The activity of xylanase was lost after passing through this column. Only 6% of the original activity and 80% of the original protein were obtained from the DEAE-cellulose column while 75-90% of the original activity and 90% of the original protein were recovered from the DEAE-Sephadex column. These results suggested that xylanase might bind strongly to the DEAE-cellulose, possibly to the cellulose matrix. Various eluants, such as high salt concentration (0.5 M $CaCl_2$ or 4 M LiCl), substrate solution (0.1-0.5% xylan), a combination of salt and substrate solutions (0.5 M $CaCl_2$, 0.5% xylan), acid or alkali solutions (pH 4-10) and hydrogen bond destabilizer (4 M urea) failed to elute the bound enzyme from the DEAE-cellulose column. The time for the elution was also varied from hours to days to make sure that it was enough for the exchange

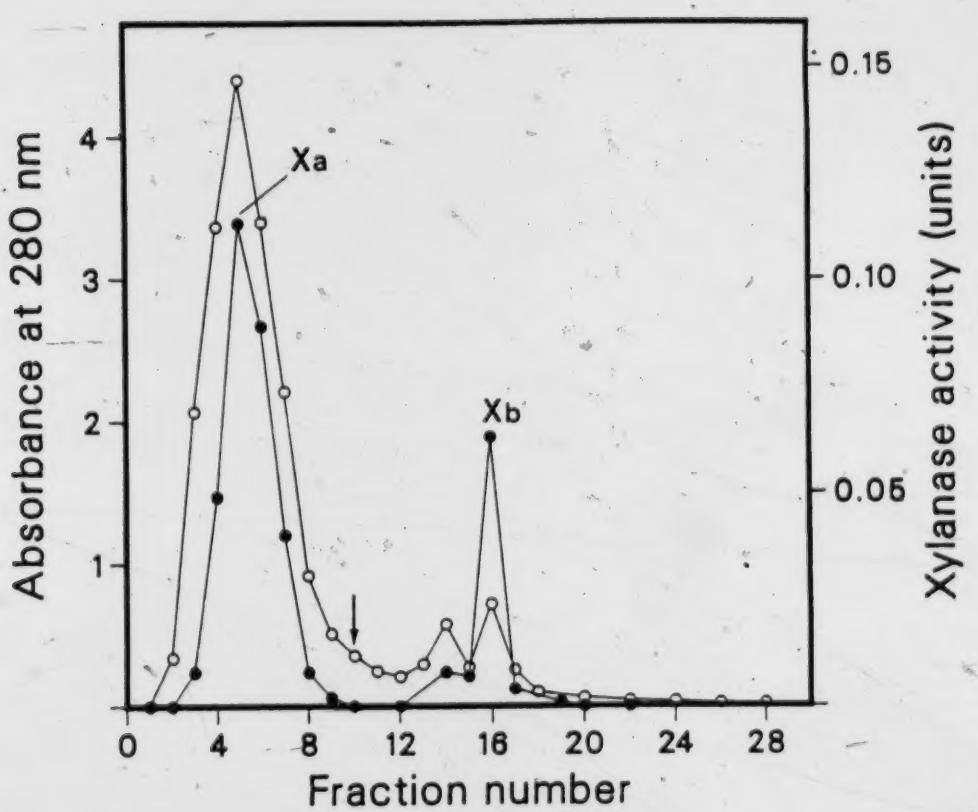



**Figure 4.** Ion exchange chromatography on DEAE-Sephadex A50 of the crude barley aleurone xylanase preparation from half-seeds. The chromatography procedures were as described for Figure 3. •-• Xylanase activity (Xa, Xb), o-o absorbance at 280 nm. The arrow indicates the application of gradient.

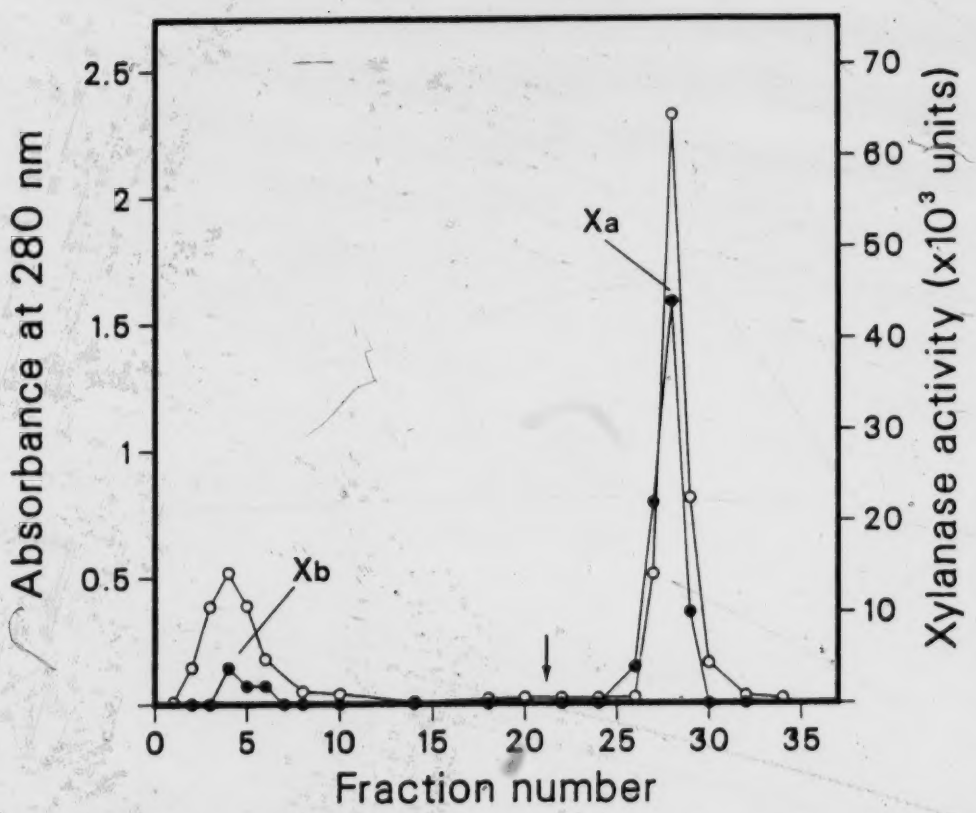



**Figure 5.** Ion exchange chromatography on CM-Sephadex C50 of the combined fractions obtained from the DEAE-Sephadex A50 column elution in Figure 4. The chromatography procedures were as described for Figure 3 except that a linear gradient of 0-0.5 M NaCl was used instead of 0.02-0.5 M $CaCl_2$ and $Ca^{2+}$ was omitted from the acetate buffer. ●-● Xylanase activity (Xa, Xb), o-o absorbance at 280 nm. The arrow indicates the application of gradient.

process. In contrast to these results, Dashek and Chrispeels (1977) found that the enzyme could be eluted from DEAE-cellulose by a salt gradient. (No details of their procedure were given.) That xylanase binds to cellulose, however, has not yet been proven. When a xylanase preparation was mixed with cellulose powder (CF 11, Whatman), it did not bind to the cellulose.

**E. Purification of barley aleurone xylanase**

As discussed earlier, the predominant form of xylanase in aleurone layers was found to be Xb, which has a pI<5.5. Thus, this enzyme was purified and characterized. In preliminary experiments, the enzyme was found to have a pI of approximately 4.6 and it appeared as a single protein band on a silver-stained two dimensional gel with an isoelectric focusing gel and SDS-PAGE as the first and second dimensional gel, respectively (results not shown). Therefore, isoelectric focusing was the method of choice for the purification of the enzyme.

The results of the enzyme purification are summarized in Table 2. The $(NH_4)_2SO_4$ precipitation did not improve the enzyme purity; however, it was used to concentrate the sample from the incubation medium. A 29-fold purification was achieved by PIEF and passage through the Sephadex G-200 column. The recovery was 45% of the initial total activity. The partial elution profile of the Sephadex G-200 column of xylanase is shown in Figure 6. Fractions 8-10 were used as the purified xylanase preparation for further studies.

The purity of xylanase is shown on the SDS-PAGE gel (Figure 7). Ten $\mu$g of protein was applied. There was only one intense protein band on this gel. One or two very faint bands with lower molecular weight (between 24 and 29 kD) were also detected but they were too faint to be visible in the photograph. The presence of these bands indicates that this enzyme preparation was not absolutely pure.

**Table 2.** Purification of barley aleurone xylanase. Barley aleurone layers were incubated in 20 mM succinic acid, 10 $\mu$M $GA_3$ and 20 mM $CaCl_2$ (pH 5.5) for 72 h at 25°C. After incubation, the enzyme from the incubation medium was purified by the steps shown below. Details on each step are given in Materials and methods.

| Step | Total Protein | Total Activity | Specific Activity | Purification | Recovery |
|---|---|---|---|---|---|
| | (mg) | (units) | (units/mg) | (fold) | (%) |
| Incubation medium | 8.44 | 2.18 | 0.259 | 1 | 100 |
| $(NH_4)_2SO_4$ precipitation | 6.15 | 1.53 | 0.249 | 1 | 70 |
| PIEF | 1.35 | 1.40 | 1.048 | 4 | 64 |
| Sephadex G-200 | 0.132 | 0.99 | 7.5 | 29 | 45 |

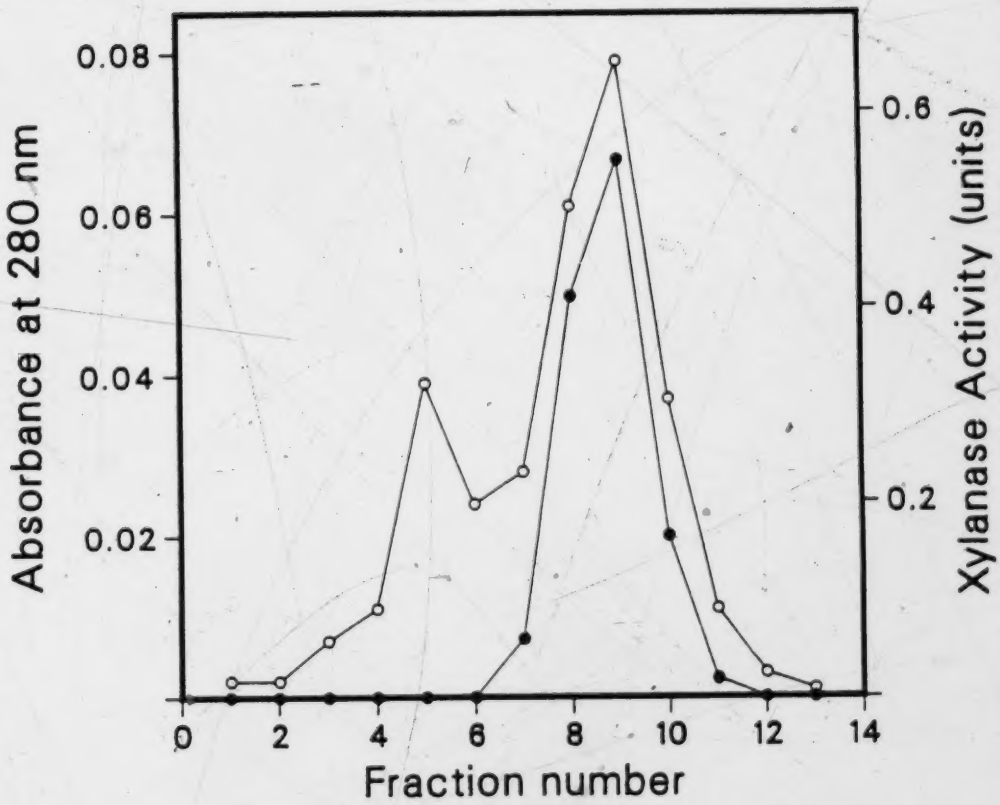
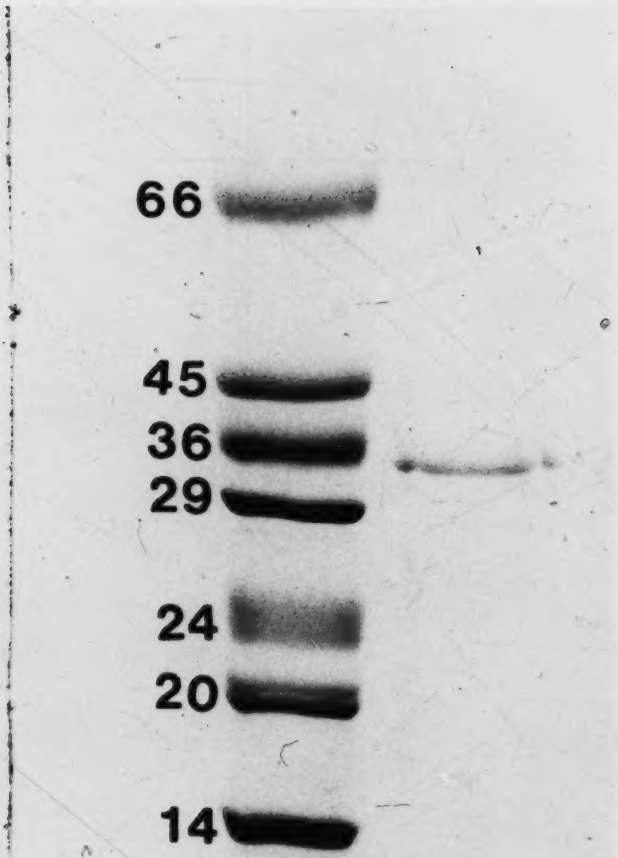



**Figure 6.** Gel filtration chromatography on Sephadex G-200 of barley aleurone xylanase after PIEF. The column (30 cm long, 1 cm diameter) was equilibrated and eluted with 20 mM potassium acetate buffer (pH 5.5). Fractions (2.0 ml) were collected at a flow rate of 3 ml/h. ●-● Xylanase activity, o-o absorbance at 280 nm.

**Figure 7.** Coomassie Blue stained SDS-PAGE of purified barley aleurone xylanase. Molecular weight markers used were (left, from the top): bovine serum albumin (66 kD), ovalbumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), trypsin inhibitor (20 kD), and α-lactalbumin (14 kD).


66
45
36
29
24
20
14

## F. Characterization of barley aleurone xylanase

*Molecular weight determinations*

The molecular weight of xylanase (shown on SDS-PAGE, Figure 7) was determined from a calibration curve (Figure 8) obtained from a plot of the relative mobility (Figure 7) against the logarithm of the molecular weight of standards (Weber and Osborn 1969). From the calibration curve, the molecular weight of xylanase was 34 kD. This figure of molecular weight was higher than the 27 kD that was obtained from the Bio-Gel P-200 gel filtration chromatography of the undenatured enzyme from crude preparation (Figure 9). Dashek and Chrispeels (1977) obtained a molecular weight of 29 kD for xylanase from a crude extract of barley aleurone layers, estimating it on a Sephadex G-100. Therefore, barley aleurone xylanase is likely to be a single polypeptide protein. The molecular weights of xylanases of fungal origin are also relatively low, ranging from 16-38 kD (Dekker and Richards 1976).

*Glycoprotein assay*

Xylanase in SDS-PAGE gave a negative reaction with the PAS stain in contrast to a positive for the ovalbumin control (results not shown). This result indicates that the aleurone xylanase is not a glycoprotein. (Xylanases from microbial sources have been reported (Dekker and Richards 1976) to be either glycosylated or non-glycosylated depending on the microorganisms and/or the isolation procedures.) It is interesting from the standpoint of protein secretion that aleurone xylanase is not a glycoprotein. Other secreted hydrolases, including barley aleurone α-amylase, have been found to be glycosylated (Akazawa and Hara-Nishimura 1985, Rodaway 1978). The results obtained here could be interpreted to indicate that aleurone xylanase might not be a secreted protein, or that it might be a secreted protein but the glycosylated part was destroyed either *in vivo* or *in vitro*. Although most secreted proteins are glycoproteins, it does not exclude the possibility that barley aleurone xylanase is a non-glycosylated secreted protein, as has also been suggested for the high pI

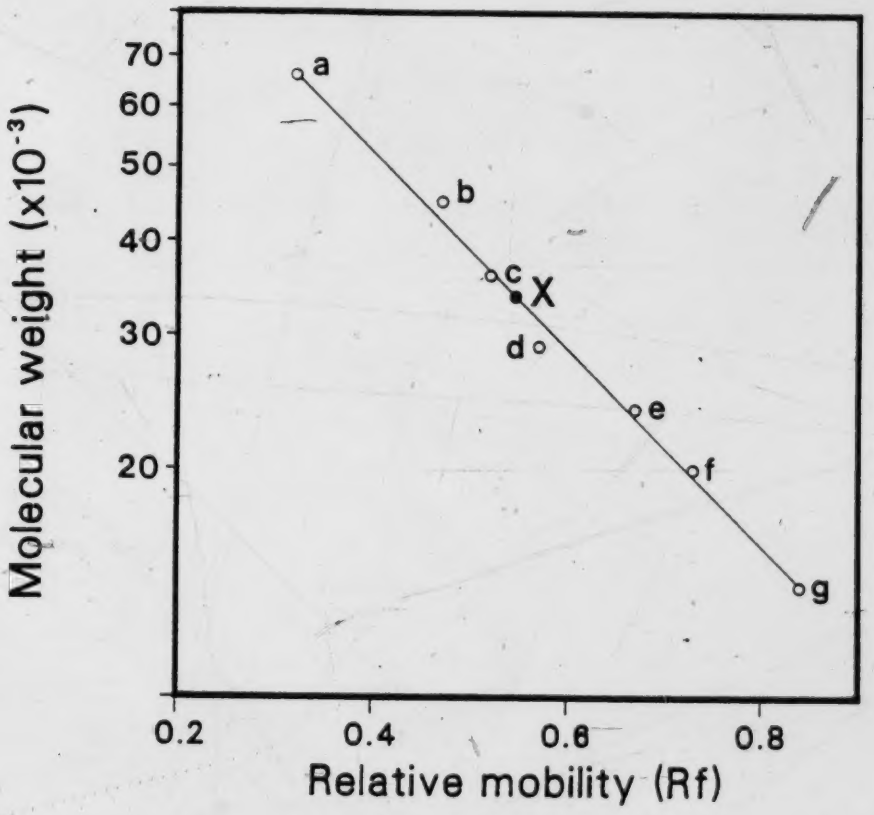



Figure 8. Determination of the molecular weight of barley aleurone xylanase from a calibration curve of a set of seven molecular weight markers on SDS-PAGE. The relative mobility of each marker and xylanase was obtained from Figure 7.

a. Bovine serum albumin (66 kD), b. ovalbumin (45 kD), c. glyceraldehyde-3-phosphate dehydrogenase (36 kD), d. carbonic anhydrase (29 kD),

e. trypsinogen (24 kD), f. trypsin inhibitor (20 kD) and g. α-lactalbumin (14 kD). X = xylanase.

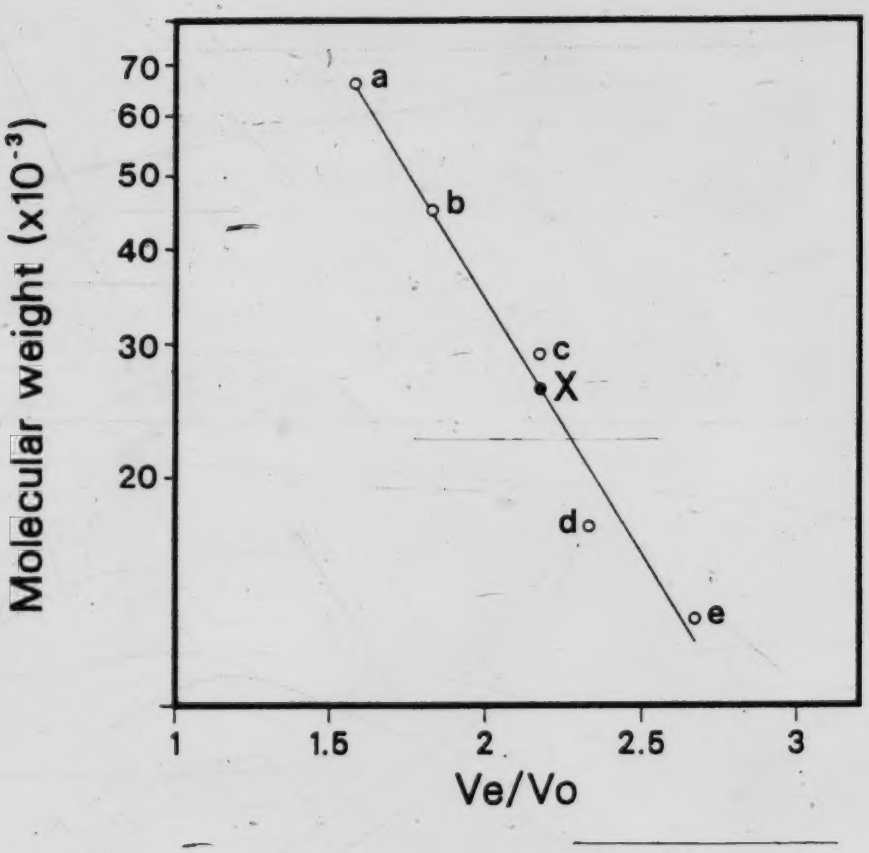



**Figure 9.** Determination of the molecular weight of barley aleurone xylanase from a Bio-Gel P-200 column (50 cm long, 0.9 cm diameter) in 20 mM succinic acid buffer (pH 5.5) 2 mM in $Ca(NO_3)_2$ with a flow rate of 3 ml/h. The molecular weight markers were: a. bovine serum albumin (66 kD), b. ovalbumin (45 kD), c. carbonic anhydrase (29 kD), d. myoglobin (17 kD) and e. cytochrome C from horse heart (13 kD). X=xylanase.

isozyme of α-amylase (Akazawa and Hara-Nishimura 1985).

*Isoelectric point determination*

The isoelectric point of xylanase was found to be pH 4.6 (Figure 10) from both analytical and preparative isoelectric focusing. Although the pH of the medium after a 3-day incubation dropped to 4.3-4.7, which was close to the pI of xylanase, there was only a negligible amount of xylanase activity in the precipitate after the medium was centrifuged. Barley aleurone xylanase (Figure 10), as well as most of the microbial xylanases, has a pI in the acidic range. However, some fungi contain also xylanases with neutral and alkaline pIs (Dekker and Richards 1976).

**G. Kinetic studies**

*Rate of xylan hydrolysis*

The rate of hydrolysis of xylan, which is routinely used as a substrate throughout this study, was linear for at least 90 min (Figure 11) under the experimental conditions used. In *Termitomyces clypeatus*, the rate of hydrolysis was shown to be exponential with time (Ghosh *et al.* 1980).

*Effects of substrate concentration on activity*

The effects of the concentration of the substrate, xylan, on xylanase activity are illustrated in Figure 12. A classical hyperbolic Michaelis-Menten curve was obtained. The Michaelis-Menten constant (Km) and maximum velocity (Vmax) obtained from the Lineweaver-Burk plot (Figure 13) were 0.86 mg xylan/ml and 0.014 units (58.3 units/mg), respectively. The barley enzyme has higher affinity for xylan than does that from mushroom (Km = 4 mg xylan/ml; Ghosh *et al.* 1980).

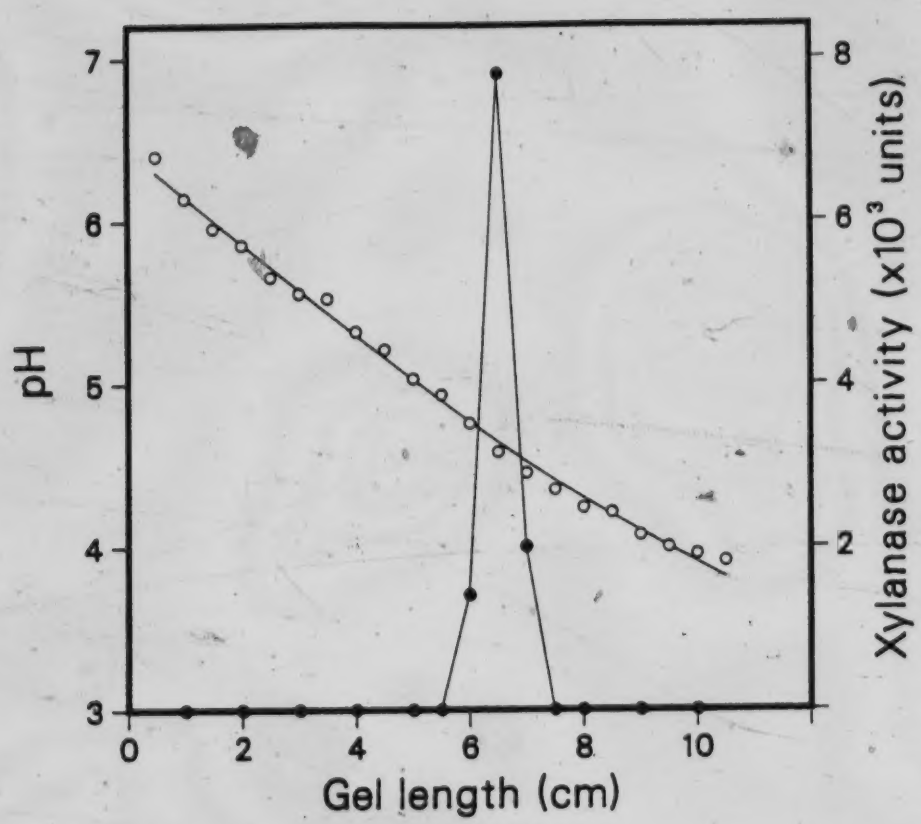



**Figure 10.** Isoelectric point determination of barley aleurone xylanase. The enzyme was run on an isoelectric focusing gel (pH 4-6) with 40 mM aspartic acid and 40 mM NaOH as the anolyte and catholyte, respectively. The gel was cut into 0.5-cm lengths and assayed for xylanase activity (●-●) and pH (o-o) as described in Materials and methods.

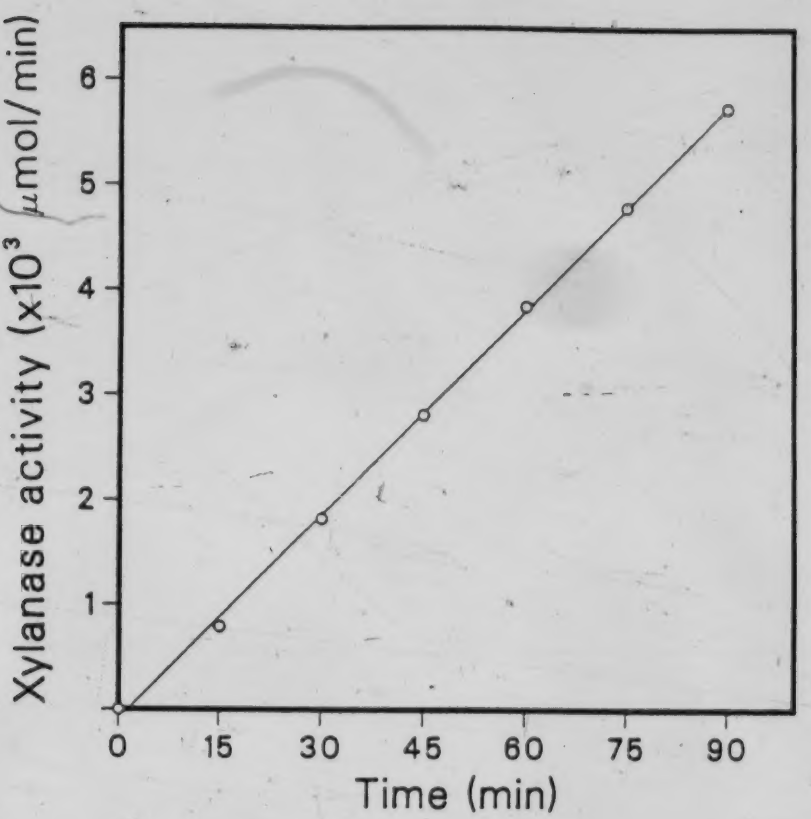



**Figure 11.** Rate of xylan hydrolysis by barley aleurone xylanase. Barley aleurone xylanase was assayed in the presence of 0.075% xylan at various times as described in Materials and methods.

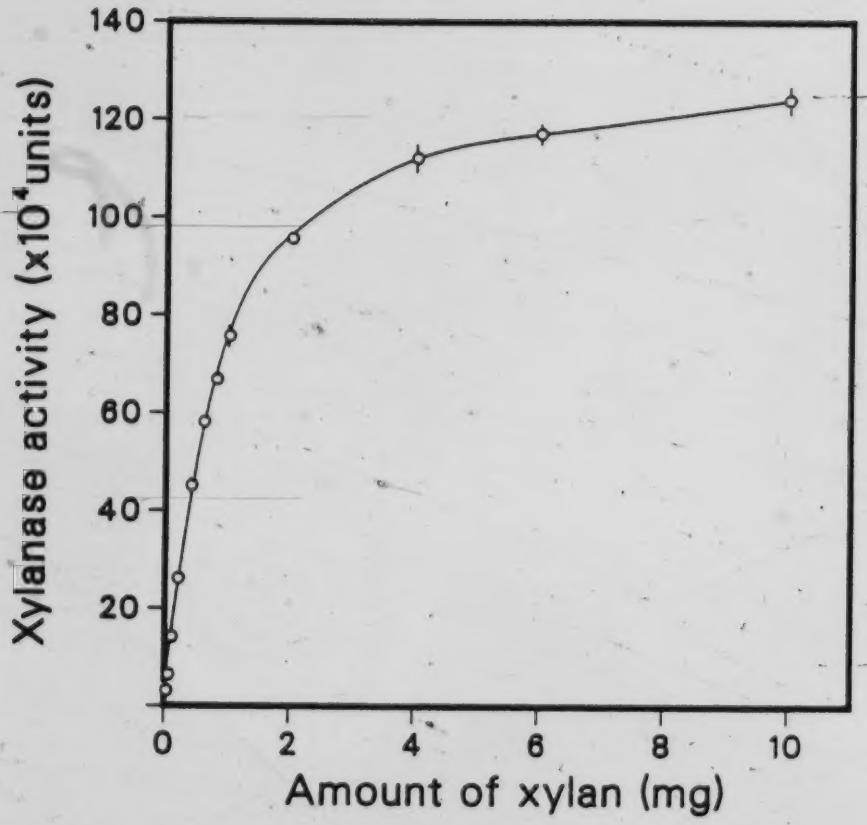



**Figure 12.** Effects of xylan concentration on barley aleurone xylanase. The activity of xylanase was assayed with various amounts of xylan (0.025-10 mg) as described in Materials and methods. Bars represent ±SD.

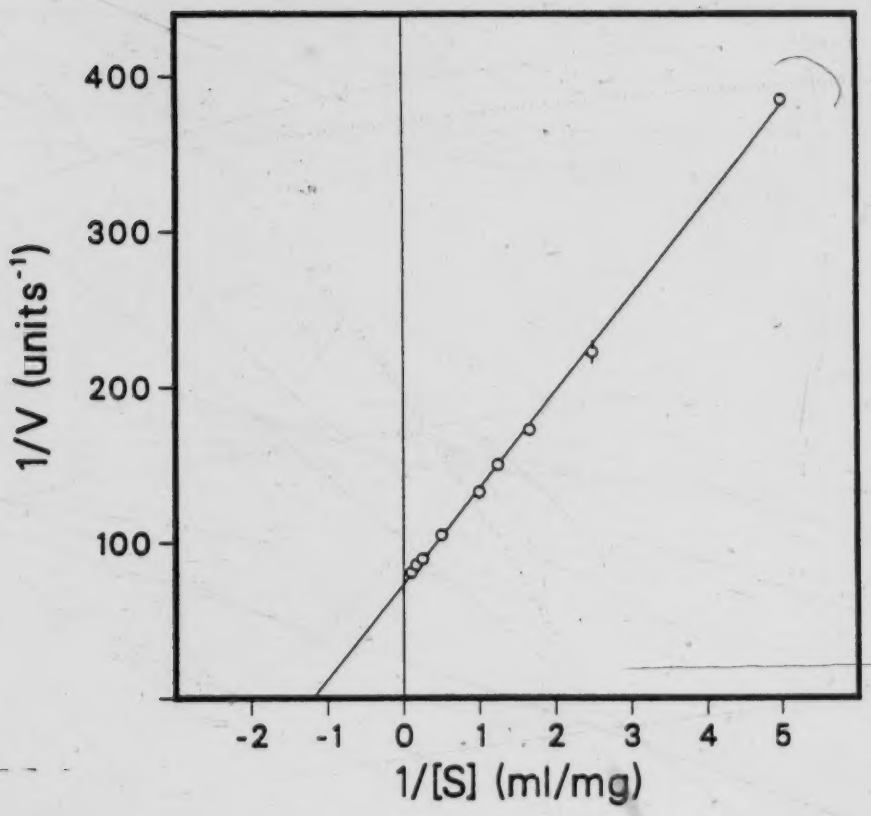



**Figure 13.** Lineweaver-Burk plot of barley aleurone xylanase activity using the data in Figure 12. Bars represent ±SD.

Xylanase produced by the fungal plant pathogen *Ceratocystis paradoxa* was also active towards sucrose and carboxymethylcellulose (CMC) (Dekker and Richards 1975b). (The authors suggested that contaminating invertase was responsible for the sucrose hydrolysis.) Barley aleurone xylanase could hydrolyze CMC as well; however, the activity towards CMC was about 6 times less than that towards xylan.

Barley aleurone xylanase bound strongly to the DEAE-cellulose, possibly to the cellulose matrix as mentioned on page 27. If xylanase bound to cellulose of DEAE-cellulose it did not hydrolyze the cellulose since no reducing products were detected. The strong binding suggests that the binding site and/or the catalytic site of xylanase for cellulose and xylan may be the same or the binding of the cellulose to enzyme may alter the binding site of xylan.

*Effects of pH on activity and stability*

The effects of pH on the activity and stability of xylanase are shown in Figures 14 and 15, respectively. The optimum pH was 5.5 for both activity and stability. There was no difference in enzyme activity in different buffers with the same pH. The activity of xylanase in 20 mM of acetate, succinate or citrate buffer, pH 5.5, was similar (a 1-2% difference, results not shown). Barley α-amylase has the same pH optimum, i.e. 5.5 (MacGregor 1978). Fungal xylanases have pH optima between 3.5-5.5 (Dekker and Richards 1976).

The pH of the incubation medium after a 3-day incubation of aleurone layers with $GA_3$ was observed to drop from 5.5 to 4.3-4.7. This observation has been previously reported (Eastwell and Spencer 1982c). The drop in pH was also observed in the absence of $GA_3$ and it may be partly caused by the production of $H^+$ via $Ca^{2+}/H^+$ exchange (Hanson 1984). However, xylanase was still stable at the low pH (Figure 15).

*Effects of temperature on activity and stability*

The effects of temperature on the activity and stability of xylanase are illustrated in Figures 16 and 17, respectively. The optimum temperature for the activity was 35°C. No

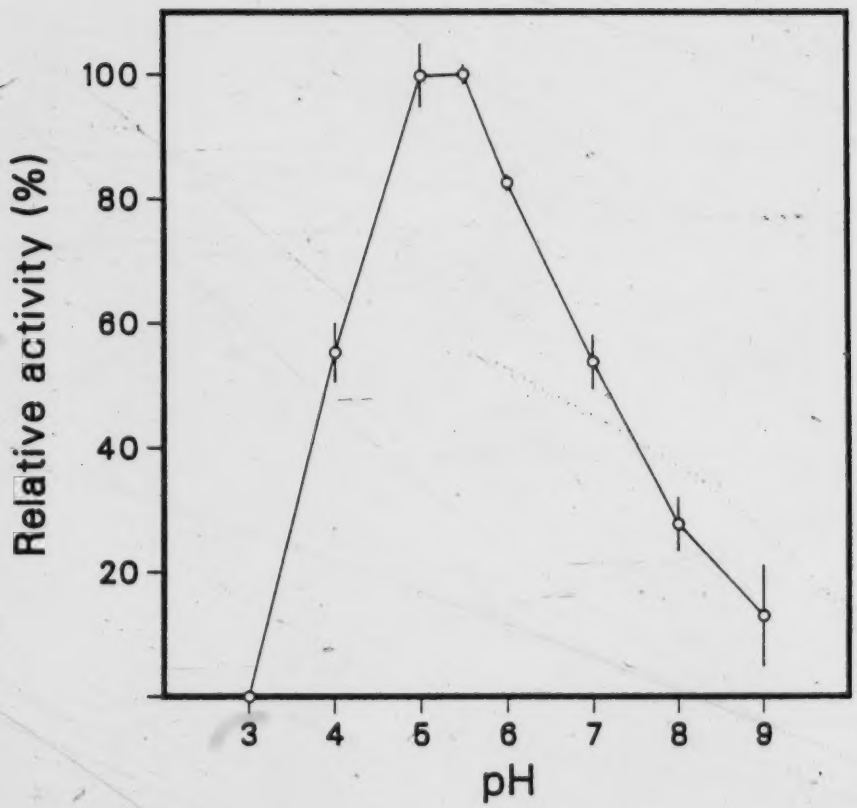



**Figure 14.** Effects of pH on barley aleurone xylanase activity. The activity of xylanase was assayed at pHs between 3.0 and 9.0 as described in Materials and methods. Bars represent ±SD.

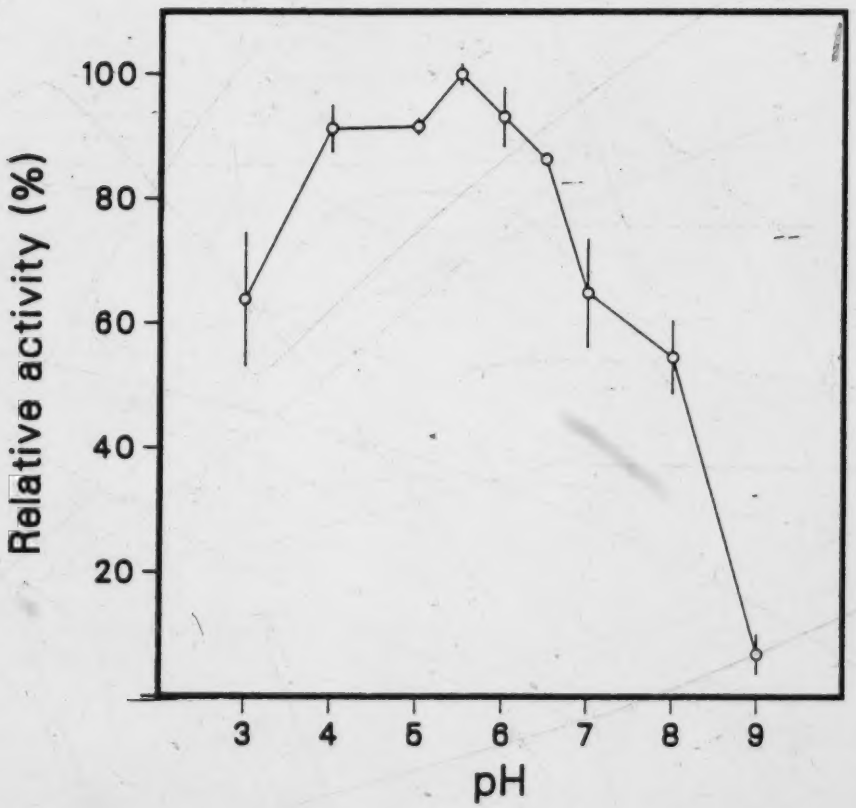



**Figure 15.** Effects of pH on barley aleurone xylanase stability. The purified xylanase was preincubated with buffers of the indicated pHs for 24 h at 4°C, and assayed for the activity as described in Materials and methods. Bars represent ±SD.

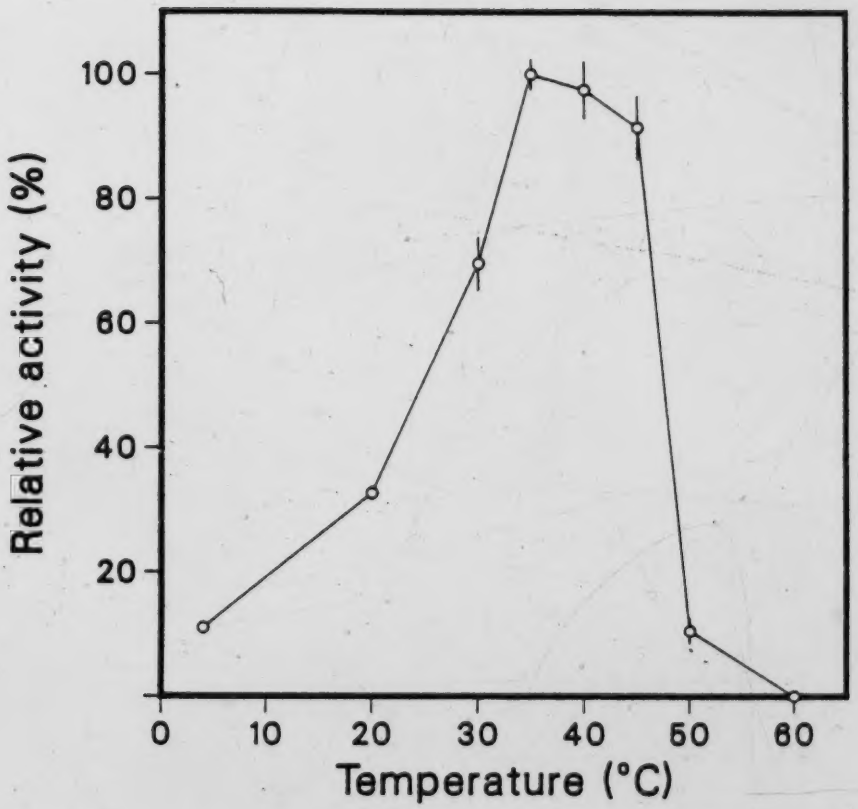



**Figure 16.** Effects of temperature on barley aleurone xylanase activity. The activity of xylanase was assayed as described in Materials and methods, at temperature between 4 and 60°C for 30 min. Bars represent ±SD.

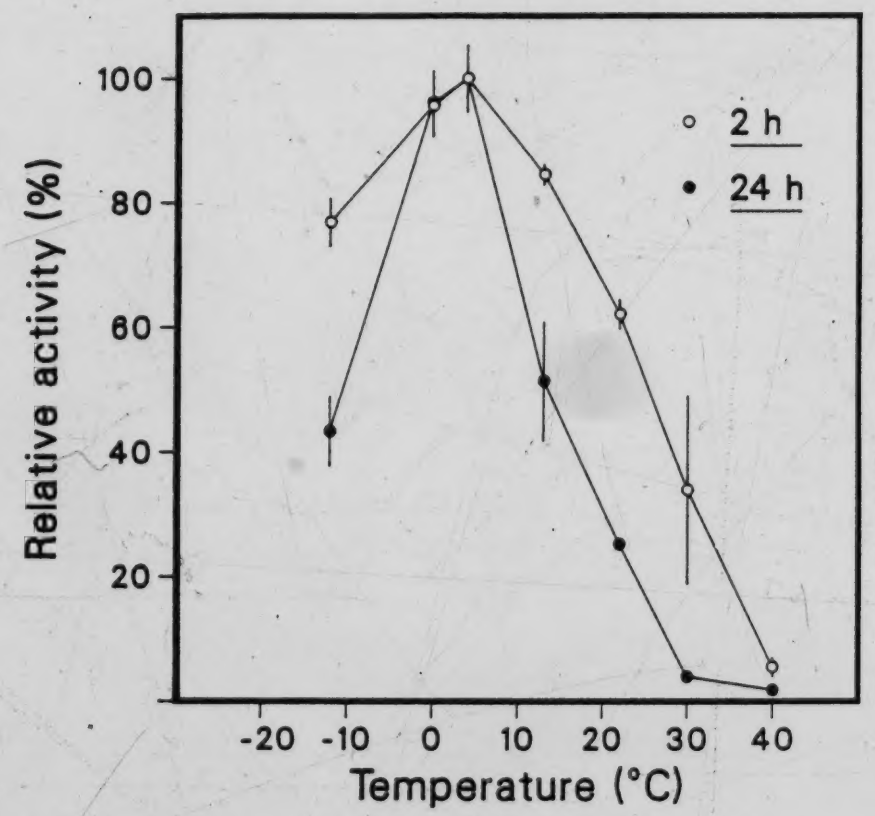



**Figure 17.** Effects of temperature on barley aleurone xylanase stability. The purified xylanase was kept in 20 mM potassium acetate buffer (pH 5.5) for 2 and 24 h at different temperature (-12 to 40°C) and assayed for activity as described in Materials and methods. Bars represent ±SD.

enzyme activity was detected at 60°C. This may reflect enzyme denaturation. Preece and MacDougall (1958) observed that endoxylanase in the crude buffer extracts of barley seeds, in the presence of calcium acetate was heat stable at 70°C for 15 min but exoxylanase was heat labile. Many fungal xylanases are thermostable and have high optimum temperatures, around 50-80°C (Dekker and Richards 1976).

Barley aleurone xylanase was quite stable. The best storage temperature was between 0 and 4°C. It should be noted that at -12°C, enzyme activity was lost (Figure 17). Calcium was found to slightly improve the stability at 4°C; however, it did stabilize the enzyme at room temperature. A 50% improvement in stability was achieved (results not shown). Therefore, $Ca^{2+}$ was included in the buffers used.

*Effects of ions and inhibitors on activity*

Among the chemicals tested (Table 3), SDS, $Hg^{2+}$, $Cu^{2+}$, $Zn^{2+}$, and $BrO_3^-$ were strongly inhibitory while $Ca^{2+}$ and $Na^+$ had no effect. Potassium bromate was demonstrated previously to inhibit barley aleurone xylanase activity as well as the release of this enzyme into the incubation medium (Eastwell and Spencer 1982c). Bromate, $Hg^{2+}$ and $Cu^{2+}$ are known as sulfhydryl enzyme inhibitors; however, the inhibitory action of bromate is different from that of $Hg^{2+}$ and $Cu^{2+}$. Bromate oxidizes thiol groups of enzymes, while $Hg^{2+}$ can react with thiol groups, the amino or imidazolium groups of histidine, peptide linkages or can co-ordinate with carboxyl and amino groups (Webb 1966). Therefore, barley aleurone xylanase may have histidine and/or acidic or basic amino acids at or near the active site. Moreover, it may require a thiol group for its catalytic activity. Ethylene glycol-bis ($\beta$-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) at 20 mM and $Cl^-$ at 40 mM inhibited xylanase activity. The inhibition by EGTA may result from the removal of some essential cations, e.g. $Ca^{2+}$. Although $Ca^{2+}$ had no effect on xylanase activity, it was found to stabilize the enzyme at room temperature as discussed earlier. The inhibition of xylanase caused by $Cl^-$ was unexpected since it has been generally used as a counter ion in most of the buffers used in this

**Table 3.** Effects of some ions and inhibitors on barley aleurone xylanase activity. Activity was assayed by incubating 0.021 units of enzyme in 10 mM potassium acetate buffer (pH 5.5), 0.75 mg xylan and ion or inhibitor (2 or 20 mM) at 30°C for 60 minutes as described in Materials and methods. The results were calculated as the percentage of the control, assayed in the absence of the ions or inhibitors. The values represent the mean of at least three different determinations ±SD.

| Chemicals | Relative enzyme activity (% of control) | |
|---|---|---|
| | 2 mM | 20 mM |
| $HgCl_2$ | 0 ± 0 | 0 ± 0 |
| $Cu(NO_3)_2$ | 0 ± 0 | 0 ± 0 |
| $Ca(NO_3)_2$ | 101 ± 4 | 104 ± 11 |
| $CaCl_2$ | 92 ± 3 | 65 ± 11 |
| $ZnCl_2$ | 35 ± 7 | 0 ± 0 |
| NaCl | 89 ± 5 | 93 ± 7 |
| $NaNO_3$ | 107 ± 13 | 102 ± 8 |
| $Na_2SO_4$ | 82 ± 4 | 95 ± 3 |
| SDS | 0 ± 0 | 0 ± 0 |
| $KBrO_3$ | 61 ± 4 | 0 ± 0 |
| EGTA | 97 ± 12 | 19 ± 5 |

research area. Nitrate (40 mM) and $SO_4^{2-}$ (20 mM) did not inhibit xylanase. In comparison, $Cl^-$ at the same concentration (40 mM) was reported to non-competitively inhibit tyrosinase activity while $NO_3^-$ and $SO_4^{2-}$ did not affect tyrosinase activity at a similar pH (i.e. 5.3). In the study on tyrosinase, steric effects were postulated to account for low inhibition with anions of large ionic radii (Krueger 1955).

None of the tested compounds markedly increased the activity of xylanase (Table 3).

**H. Role of xylanase in cell wall degradation and enzyme release**

The release of enzymes from aleurone cells into the starchy endosperm was suggested by Varner and Mense (1972) to be composed of two phases, namely, the active secretion of the enzymes across the plasmalemma and the diffusion release of the enzymes through the cell walls. In the presence of $GA_3$, the release of enzymes accompanies the degradation of aleurone cell walls (Ashford and Jacobsen 1974, Jones 1969c). Ashford and Jacobsen (1974) have proposed that the diffusion of the enzymes through the walls cannot occur unless the walls are hydrolyzed. It was of interest to investigate the role of xylanase with respect to the degradation of the largely arabinoxylan walls of the aleurone layers, and the release of other enzymes from the layers.

*Time course study of xylanase production*

It has been established that release of α-amylase begins between 5-8 h after incubation of aleurone layers with $GA_3$ (Ashford and Gubler 1984). Xylanase may play a role in this initial release process if it is present in the layers before or during this period. However, the evidence in the literature for the presence of xylanase during this time is not conclusive. Taiz and Honigman (1976) could detect xylanase at zero time but Dashek and Chrispeels (1977) and Eastwell and Spencer (1982b) could not detect any xylanase activity until 24 and 12 h of incubation, respectively. The high concentration of NaOCl used to disinfect the seeds by these workers can lower the levels of xylanase production as discussed earlier. Variations in seed lots

are known to occur. Also xylanase activity is difficult to assess because very low levels are produced by aleurone layers in response to $GA_3$. In order to overcome this difficulty, we used low concentration of NaOCl to sterilize the seeds, a larger number of layers (10-50) and concentrated the samples by use of microconcentrator to increase the sensitivity of detection.

Xylanase was detected in layers prior to exposure to $GA_3$. At zero time, a very low but consistent concentration (0.11 munits/layer, absorbance at 520 nm $>$ 0.1) of xylanase was detected in the layers (Figure 18). In the absence of $GA_3$, xylanase levels increased with the incubation time; however, the total activity of xylanase was 5-19 times less than that in the presence of $GA_3$. Therefore, $GA_3$ did induce an increase in xylanase activity. The increase of xylanase in the absence of $GA_3$ may result from endogenous $GA_3$ (Akazawa and Hara-Nishimura 1985, Ashford and Gubler 1984, Atzorn and Weiler 1983, Chrispeels and Varner 1967). Xylanase accumulated within the layers before it did so in the medium. After 48 h, xylanase levels in the layers decreased. However, this decrease was accompanied by an increase in levels of xylanase released into the medium and the activity continued to increase for the 96 h measurement period. Figure 19 shows the progressive increases in xylanase activity in the layers and $\alpha$-amylase in the medium in the presence of $GA_3$. Xylanase activity in the layers began to increase at the same time as $\alpha$-amylase activity in the medium, after approximately 6 h incubation with $GA_3$. Similar results were also obtained by Taiz and Honigman (1976), but the distribution of enzyme between the layers and the medium at the first 20 h of incubation was different. This may be explained by the use of different material, i.e. they used isolated layers and we used layers with seed coats attached. With the latter, we have found less enzyme is released because of obstruction by the seed coat (results not shown).

The changes in the concentrations of inorganic ions, $PO_4^{3-}$, $Mg^{2+}$, and $K^+$, in the medium and layers during incubation in the presence or absence of $GA_3$ are illustrated in Figure 20. The levels of these ions in the medium increased in response to $GA_3$ and the increase was at about the same time as xylanase activity increased in the layers. The release of

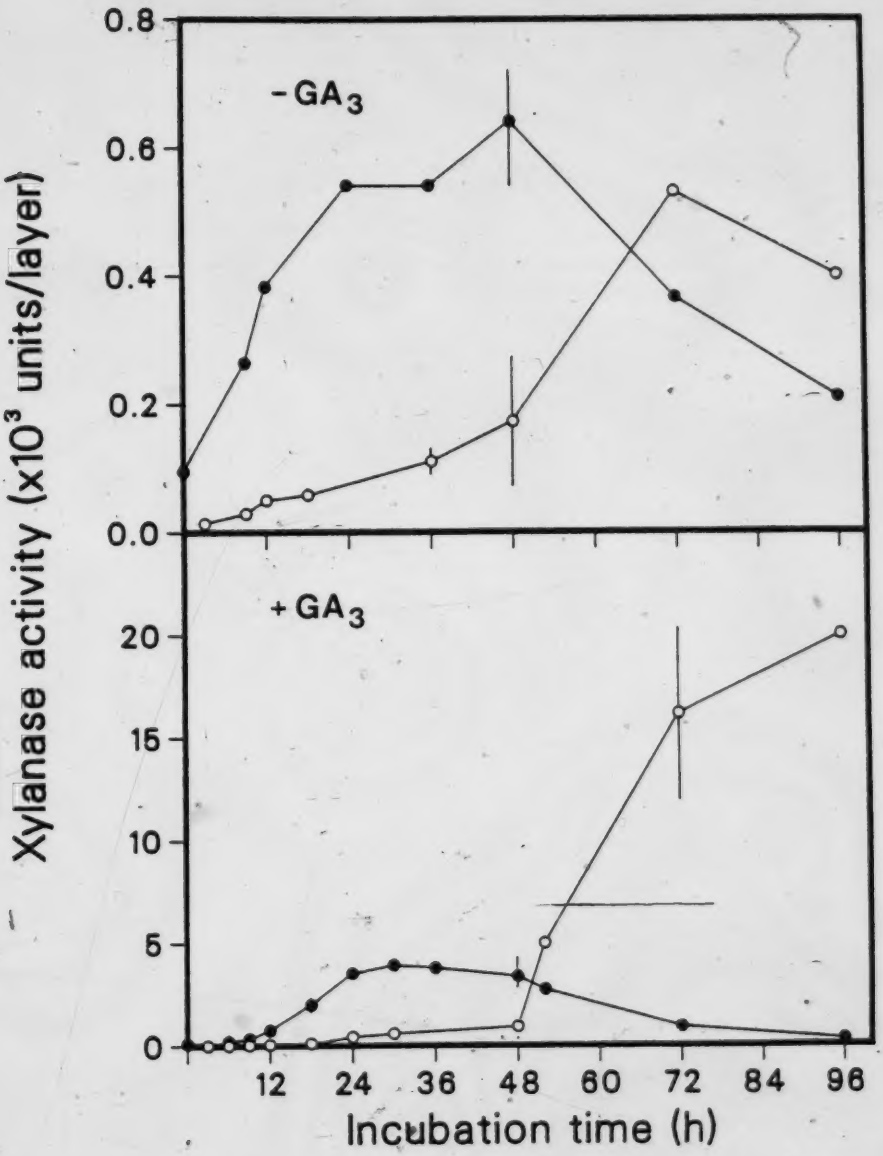



**Figure 18.** Time course of xylanase activity in barley aleurone layers (●-●) and incubation medium (o-o) in the absence or presence of 10 μM GA, at 22°C. The incubation medium was 20 mM succinic acid buffer (pH 5.5) 20 mM in $Ca(NO_3)_2$. Bars represent ±SD.

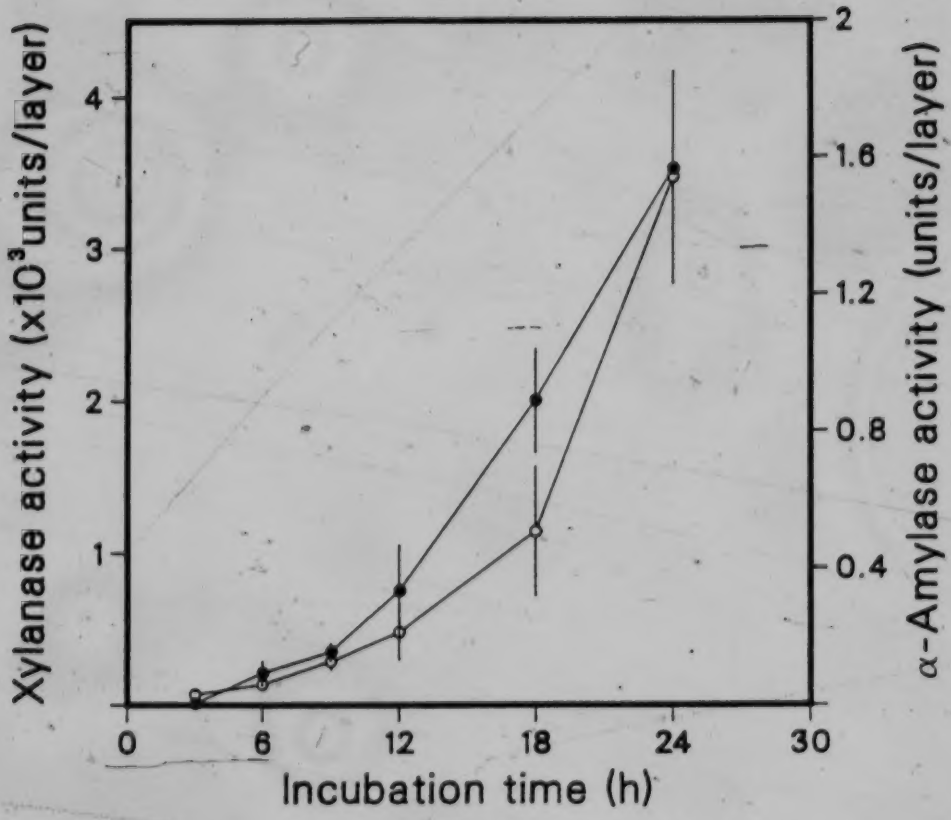



**Figure 19.** Time course of xylanase activity in barley aleurone layers (●-●) and α-amylase in the incubation medium (o-o) in the presence of 10 μM $GA_3$ at 22°C. The incubation medium was as in Figure 18. Bars represent ±SD.

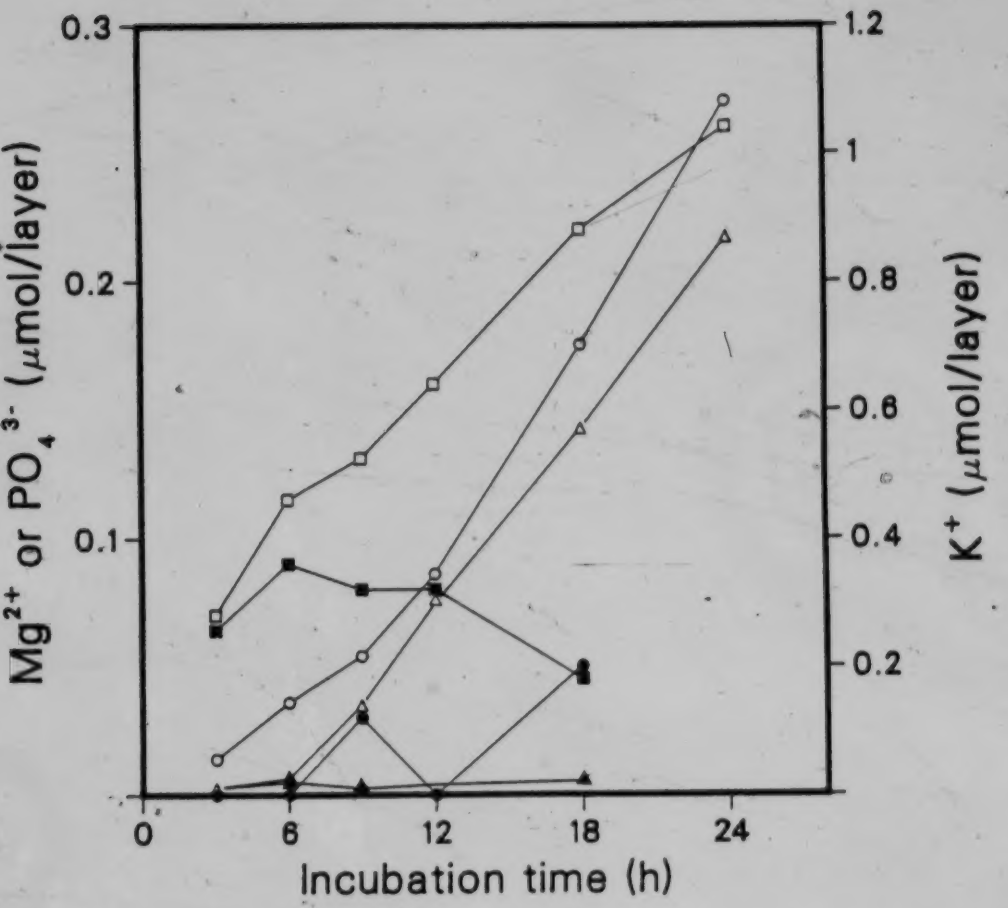



Figure 20. Time course of the appearance of $K^+$, $Mg^{2+}$ and $PO_4^{3-}$ in the incubation medium when barley aleurone layers were incubated in the absence (closed) or presence (open) of 10 $\mu$M GA, at 22°C. The incubation medium was as in Figure 18. △-△ $K^+$, □-□ $Mg^{2+}$, o-o $PO_4^{3-}$.

these ions into the medium may partly result from the removal of barriers in the cell walls as these become degraded. This finding corroborates the results of Jones (1973b) on time course of $PO_4^{3-}$, $Mg^{2+}$ and $K^+$ release in the barley aleurone system.

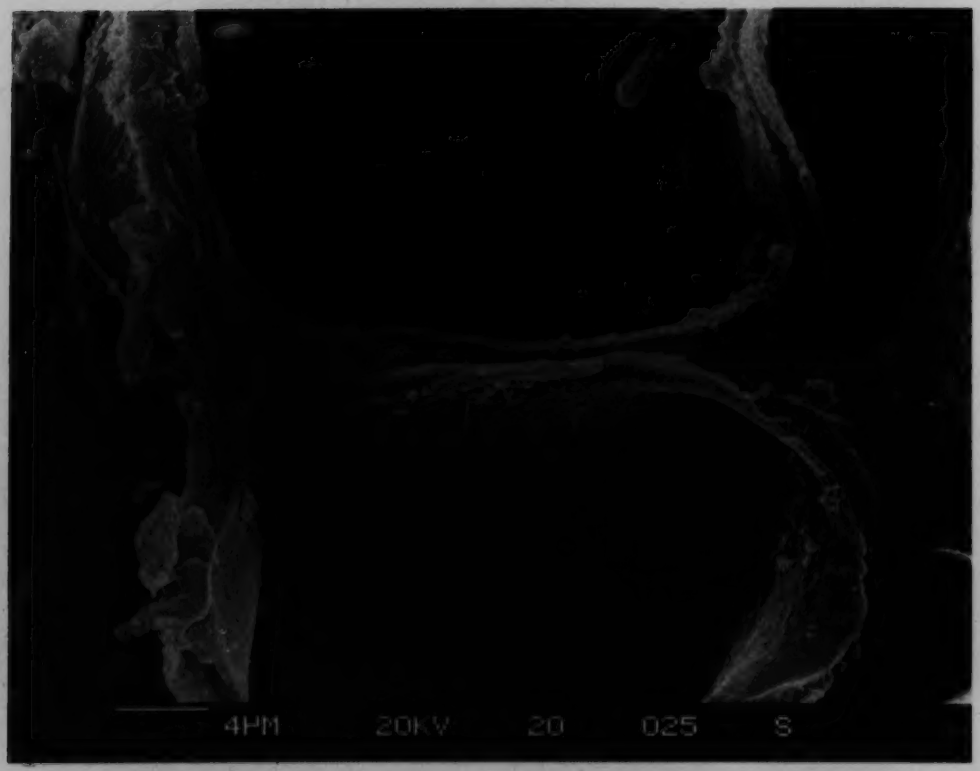
*Effects of purified xylanase on aleurone cell walls*

Although a crude preparation of aleurone xylanase has been demonstrated to hydrolyze arabinoxylan (Taiz and Honigman 1976), no evidence has been provided until now for a role of xylanase in cell wall degradation. Scanning electron micrographs of aleurone layers after 4, 20, and 50 h incubation with the purified xylanase (Figure 21) show that after 20 h incubation, the walls of the aleurone cells in control layers were still intact compared with the degraded cell walls of layers incubated with purified xylanase (Figure 21c, d). The hydrolysis of the cell walls in the treated layers can be observed (Figure 21d) as a groove in the region between two cells. In the control layers (Figure 21c) a groove was not evident. (The rough surface is a result of preparation process, not hydrolysis.) Cell walls appeared to be more hydrolyzed after 50 h of incubation (Figure 21f). The hydrolysis of cell walls began from the area of the middle lamella (Figure 21d, 22). Hydrolysis of the walls at the corners of the cells was also observed (Figure 22). The same pattern of hydrolysis of aleurone cell walls was also found in barley aleurone layers treated with $GA_3$ (Ashford and Jacobsen 1974). Jones (1972) also observed that the digestion of the walls begins at the area surrounding the plasmodesmata.

The layers incubated for 4 h did not show any signs of hydrolysis (Figure 21a, b). This absence of visible cell wall degradation at 4 h, however, does not exclude the possibility of wall hydrolysis since the sensitivity of scanning electron microscopy is limited. Other workers, when they incubated the aleurone layers with $GA_3$, reported variation in the time of the first detection of cell wall degradation. The time varied from 8-16 h (Briggs 1973, Jones 1972).

**Figure 21.** Scanning electron micrographs of barley aleurone layers after 4, 20 and 50 h incubation in 20 mM potassium acetate (pH 5.5) 2 mM in $Ca(NO_3)_2$ at 25°C in the absence or presence of 0.10 units of purified barley aleurone xylanase. a, c and e: control layers at 4, 20 and 50 h, respectively. b, d and f: treated layers at 4, 20 and 50 h, respectively. Bars = 1 μm.

**Figure 22.** Scanning electron micrograph of barley aleurone layer after 20 h incubation in 20 mM potassium acetate (pH 5.5) 2 mM in $Ca(NO_3)_2$ at 25°C in the presence of 0.10 units of purified barley aleurone xylanase. Bars = 4 μm.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Based on the direct scanning electron micrograph observations and xylanase activity present in the layers prior to the release of other hydrolases, the extent of aleurone cell wall hydrolysis by xylanase can be calculated as follows:

The thickness of aleurone cell wall = 1.5 $\mu$m (average from 4 cells from scanning electron micrographs). This figure for wall thickness is similar to the average wall thickness (2 $\mu$m) from Bacic and Stone (1981a). A much thicker wall (10-30 $\mu$m) was reported by Jones (1969a); however, we obtained about 1.3 $\mu$m thick wall by using his raw data (an electron micrograph of aleurone cells).

If the aleurone cell is cubic and has an average side of 20 $\mu$m (from the scanning electron micrographs), the inner volume of the aleurone cell (without cell wall) = 8,000 $\mu m^3$.

Volume of the whole cell (with cell wall) = $(20+3)^3$ = 12,167 $\mu m^3$

Thus, volume of the cell wall = 12,167 — 8,000 = 4,167 $\mu m^3$

If the cell wall has a density = 1.5 g/ml (average from the densities of xylose, sugar and starch, which are 1.5, 1.6 and 1.5 g/ml, respectively (CRC handbook of chemistry and physics 1971, The Merck index 1976) and the density of a cell = 1 g/ml, then the amount of arabinoxylan in the cell wall in each layer (Ac) is:

$$Ac = d.f.w.v \qquad (1)$$

where d = density of cell wall material

f = arabinoxylan fraction of cell wall material which is 85% (McNeil *et al.* 1975)

w = dry weight of one layer = 0.52 mg

v = fraction volume of cell wall compared to entire cell

Thus, amount of arabinoxylan in each layer = 1.5 x 0.85 x 0.52 x 4,167 / 12,167 = 0.23 mg

Between 3 and 6 h incubation with $GA_3$, there were 0.13 and 0.25 munits of xylanase in each layer, respectively, which could hydrolyze approximately 2-6% of the cell wall. This amount of wall hydrolysis is probably enough to permit the release of enzymes and other

solutes secreted by the cells. Caution must, of course, be observed in extending results from *in vitro* experiments to *in vivo* situations. Ashford and Jacobsen (1974) provided evidence that release of acid phosphatase occurs through channels in the aleurone cell walls. If hydrolysis by xylanase is concentrated at such pores, the above percentages would be even more effective in enabling exit of enzymes.

Xylanase is present in the layers at an activity of about 4 munits/layer at 24-36 h. By this time, the activity of $\alpha$-amylase is found primarily in the medium, while that of xylanase is located primarily in the layers. It is possible that xylanase is bound to its substrates in the cell wall. The strong binding of xylanase to the substrate may assist the enzyme, which is small in amount, to hydrolyze the substrate more efficiently during the early stages of germination. Xylanase can bind strongly to DEAE-cellulose but not DEAE-Sephadex. This might imply that xylanase can bind to cellulose in the cell wall. Apart from the major cell wall polysaccharide, arabinoxylan, cellulose and $\beta$-1,3; 1,4-glucans are found in the aleurone cell wall (Bacic and Stone 1981b, McNeil *et al.* 1975). All of these wall polysaccharides may serve as binding compounds for xylanase since they all have $\beta$-1,4-linkages.

Xylanase activity in the medium increases dramatically (Figure 18) after 48 h. By this time, a 60% loss of the cell wall pentose has been shown to occur (Dashek and Chrispeels 1977). It is interesting to consider why there is such a large amount of xylanase after the aleurone cell wall has been degraded and the release of other hydrolytic enzymes has reached a plateau (Ashford and Jacobsen 1974, Eastwell and Spencer 1982a, Jacobsen and Varner 1967, Jones 1971, Taiz and Honigman 1976). The endosperm cell wall has similar polysaccharides to that of the aleurone cell wall but there is 25% arabinoxylan in endosperm cell wall as opposed to 85% in aleurone cell wall (Ballance and Manner 1978, Fincher 1975, Fincher 1976). Moreover, it has been observed that the breakdown of endosperm cell wall precedes the attack on starch (Dickson and Shands 1941, Morrall and Briggs 1978). This observation indicates the importance of cell wall degradation to the mobilization of nutrients in endosperm. Therefore, xylanase from the aleurone tissue may also hydrolyze the endosperm cell wall since there is no

xylanase produced by the starchy endosperm (results not shown). The hydrolysis of endosperm cell wall by xylanase might facilitate the entry of hydrolytic enzymes and/or supply sugars (xylose, arabinose and their products) as energy sources and/or for conversion to components of polymeric compounds (e.g. nucleic acids) in the growing embryo (Morrall and Briggs 1978, Stone 1985). In addition, xylanase might also serve as an important factor in producing cell wall hydrolysis products that may serve as substrates in biosynthetic reactions and respiration in both the seedling and in barley-fed animals (Stone 1985).

**I. Gibberellic acid and xylanase synthesis**

Gibberellic acid has been suggested to play an important role in the initiation of carbohydrate and lipid metabolism in cereal seeds by inducing the synthesis of many enzymes for starch degradation, $\beta$-oxidation of fatty acids (Laidman 1983) and lecithin biosynthesis (Johnson and Kende 1971). The increases in the activity of hydrolases in response to GA were proposed to result from the *de novo* synthesis, not from the activation of existing enzyme precursors (Briggs 1963). Barley aleurone $\alpha$-amylase and protease have been shown to be synthesized *de novo* in response to $GA_3$ (Filner and Varner 1967, Jacobsen and Varner 1967, Varner *et al.* 1965). In the following experiments, cycloheximide, which inhibits protein synthesis, cordycepin, which inhibits RNA synthesis, and a density labeling technique (Hu *et al.* 1962) were used to investigate the effects of $GA_3$ on the synthesis of xylanase.

*Effects of cycloheximide and cordycepin on xylanase synthesis*

Cycloheximide is known as an inhibitor of protein synthesis in eukaryotic systems. It inhibits at the translocation step by interacting with the 60s ribosomal subunit (Vazquez *et al.* 1982). Cycloheximide at 10 $\mu$g/ml inhibited the synthesis of barley aleurone xylanase almost completely, i.e. 99 and 98% inhibition was observed when added at 0 and 12 h, respectively, after $GA_3$ application (Table 4). These results suggest that there was no *de novo* xylanase synthesis to 12 h. However, the time course study of xylanase (page 52 and Figure 18)

**Table 4.** Effects of cycloheximide and cordycepin on the synthesis of barley aleurone xylanase. Ten aleurone layers were incubated for 72 h at 25°C in 2 ml of 20 mM succinic acid, 10 $\mu$M $GA_3$ and 20 mM $Ca(NO_3)_2$ in the absence or presence of 10 $\mu$g/ml cycloheximide or cordycepin. The application of cycloheximide or cordycepin was at either 0 or 12 h of incubation. Xylanase in the incubation medium and in the layer extract was prepared and assayed as described in Materials and methods. The values represent the mean of at least four different determinations ±SD.

| Inhibitor & Treatment time | Xylanase activity (munits/layer) | |
|---|---|---|
| | Medium | Extract |
| — | 16.14 ± 4.18 | 0.89 ± 0.20 |
| cycloheximide 0 h | 0.14 ± 0.04 | 0 ± 0 |
| cordycepin 0 h | 0.19 ± 0.07 | 1.87 ± 0.35 |
| cycloheximide 12 h | 0.10 ± 0.11 | 0.24 ± 0.03 |
| cordycepin 12 h | 2.44 ± 0.89 | 1.06 ± 0.45 |

showed that there was an increase in xylanase levels from 0 to 96 h after exposure to $GA_3$. This increase in xylanase levels in response to $GA_3$ may result from the activation of a preexisting precursor of xylanase by $GA_3$. However, another possibility is that xylanase may be synthesized *de novo* but the turnover rate of the enzyme may be high and/or the enzyme may be less stable in the presence of cycloheximide. (After application of cycloheximide, the incubation was continued to complete 72 h incubation.)

Cordycepin, which inhibits both the transcription of, and poly(A) addition to RNA (Suhadolnik 1979) also inhibited 88 and 80% of the synthesis of barley aleurone xylanase when added at 0 and 12 h, respectively, after $GA_3$ application (Table 4). When cordycepin was added at 12 h, there was a small increase (8%) in the xylanase synthesis compared with zero time.

The data in Table 4 suggest that there is preexisting mRNA for xylanase at zero time since when cordycepin was added at that time, 2.06 munits of xylanase/layer was detected, i.e. the synthesis of xylanase arose from the translation of the preexisting mRNA. (This assumes complete effectiveness of cordycepin in the inhibitions mentioned above.) This interpretation is partially based on the data from the cycloheximide treatment at zero time, which indicates that there was only 0.14 munits of xylanase/layer. The xylanase responsible for the activity of 0.14 munits/layer was likely preexisting enzyme. As discussed on page 52, there was 0.11 munits of xylanase/layer in the layers prior to exposure to $GA_3$. Therefore, cycloheximide could turn off xylanase synthesis immediately and completely when it was added. These results indicate that all of the xylanase activity induced by $GA_3$ requires the synthesis of new xylanase. Cycloheximide inhibits about 95% of α-amylase and protease synthesis in the barley aleurone system (Jacobsen and Varner 1967). We obtained about 93% inhibition of α-amylase by cycloheximide (results not shown). Although cordycepin, added at 12 h after $GA_3$ application, was less inhibitory than when added at zero time after $GA_3$ incubation, it still inhibited about 80% of the total xylanase synthesis. This result implies that the synthesis of xylanase mRNA was still progressing at 12 h of incubation with $GA_3$ and/or that xylanase

mRNA was unstable. In comparison, synthesis of the mRNA for α-amylase and protease has been found to be complete by 12 h incubation with $GA_3$, since the addition of cordycepin at this time did not inhibit the synthesis of these two enzymes (Ho and Varner 1974). We found that 85% of α-amylase activity was obtained when cordycepin was added at 12 h after $GA_3$ (results not shown). It was suggested by Ho and Varner (1974) that α-amylase mRNA was quite stable. Cordycepin has been reported to inhibit the incorporation of uridine into total RNA to the same extent at either 0 or 12 h after incubation with $GA_3$, and to inhibit 50% of total protein synthesis (Ho and Varner 1974).

*De novo synthesis of xylanase*

The density labeling originally came from the study of DNA replication *in vivo* (Meselson *et al.* 1957, Meselson and Stahl 1958) and was first applied, by Hu *et al.* (1962), to the study of protein synthesis. The principle behind this technique is that when the tissues are supplied with a compound containing a heavy isotope that can be incorporated into amino acids used for protein synthesis, the newly synthesized protein will be heavier than the preformed protein. In the barley seed system, the density labeling technique has been used to demonstrate the *de novo* synthesis of many enzymes, namely, α-amylase (Filner and Varner 1967, Hardie 1975), protease (Jacobsen and Varner 1967), peroxidases (Anstine *et al.* 1970), β-1,3-glucanase and ribonuclease (Bennett and Chrispeels 1972), acid phosphatase (Chrispeels and Varner 1973), and limit dextrinase and α-glucosidase (Hardie 1975). The source of heavy isotope was either deuterium oxide ($D_2O$) or $H_2{}^{18}O$. The D- or $^{18}O$-labeled amino acids were derived from the hydrolysis of reserve proteins in aleurone grains (or protein bodies) in the presence of $D_2O$ or $H_2{}^{18}O$ (see equations 2 and 3).

$$\text{protein} + D_2O \longrightarrow RCHNHDCOOD \qquad (2)$$

$$\text{protein} + H_2{}^{18}O \longrightarrow (RCHNH_2C^{16}O^{18}OH \leftrightarrow RCHNH_2C^{18}O^{16}OH) \qquad (3)$$

The D- or $^{18}O$-labeled amino acids were then incorporated into the newly synthesized protein, which would in turn exhibit higher buoyant density than that with unlabeled amino acids. By

using isopycnic equilibrium sedimentation in CsCl gradient, the two proteins with different buoyant density could be distinguished.

In the present experiment, when barley aleurone layers were incubated with succinate buffer, $GA_3$ and $Ca(NO_3)_2$ prepared in $D_2O$, $H_2O$ or $H_2{}^{18}O$, after 72-h period of incubation, distinct differences in the appearance of the layers were observed. The layers from the $D_2O$ preparation looked different from those from the $H_2O$ or $H_2{}^{18}O$. The layers from the $D_2O$ appeared unchanged or 'thick' as opposed to the 'thin' look of the layers of those from $H_2O$ or $H_2{}^{18}O$. In normal experiments, 'thin' layers were always obtained. The 'thin' look of the layers may be a sign of dying cells, which may result from the hydrolysis of the protein bodies and/or cell walls. The xylanase activity obtained from the preparation in 75% $D_2O$ was about 7 times less than that from the preparation in $H_2O$, and 5 times less than that from the preparation in 80% $H_2{}^{18}O$. The inhibition by $D_2O$ of acid phosphatase and peroxidase synthesis in barley seeds has also been reported (Anstine *et al.* 1970, Chrispeels and Varner 1973). At 75% $D_2O$, the growth of barley embryo was inhibited (Anstine *et al.* 1970). It is interesting that the plant is sensitive to isotopic change. Some properties of $D_2O$ are different from those of $H_2O$ (e.g. density, boiling point etc.). On the other hand, xylanase activity obtained from the 80% $H_2{}^{18}O$ preparation was about 70% of that from $H_2O$. Therefore, $H_2{}^{18}O$ is the better label when compared to $D_2O$. However, the high cost of $H_2{}^{18}O$ is a limiting factor (275 times higher than $D_2O$). The period of incubation for this experiment was extended to 120 h instead of 72 h in order to get enough enzyme from the $D_2O$ preparation.

In preliminary experiments, CsCl was found to both inhibit the xylanase activity and quench the radioactivity. Cesium chloride at 25% saturation inhibited about 10-20% of the xylanase activity. (The concentration of CsCl distributed in the CsCl gradient was about 23% at the top and 40% at the bottom.) However, the effect of CsCl on the xylanase assay in the density labeling experiment was negligible since it was diluted (20 times) with the substrate solution. The quenching of radioactivity by CsCl was reduced by adding water before the radioactivity determination, as suggested previously (Chrispeels and Varner 1973).

The results of the density labeling in $D_2O$ or $H_2{}^{18}O$ compared to that in $H_2O$ are shown in Figures 23 and 24, respectively. In each figure, the position of the radioactive marker ovalbumin was lined up to compare the position of xylanase. The distribution of the radioactivity and xylanase activity in each experiment was similar and close to the normal curve. A slight deviation from the normal curve was observed. This may result from the heterogeneity of xylanase and/or the disturbance of the fraction during the collection. The deviation from the normal curve also occurred in the marker. The density of the marker as well as the xylanase was determined by averaging the midpoints of the peak at two different peak heights (1/2, 3/4 peak height). The refractive indices were converted to density using a refractive index table (CRC Handbook of biochemistry 1970).

The marker and xylanase in $H_2O$ banded at densities of 1.2942 and 1.3075 g/ml, respectively (Table 5). The densities of xylanase in the experiments with $D_2O$ and $H_2{}^{18}O$ were 1.3218 and 1.3191 g/ml, respectively (Table 5). The density shifts of xylanase in $D_2O$ and $H_2{}^{18}O$ were 0.0143 and 0.0116 g/ml, or 1.1 and 0.9%, respectively. Hu *et al.* (1962) calculated the maximum density shifts in the presence of 75% $D_2O$ and 80% $H_2{}^{18}O$ would be 0.045 and 0.024 g/ml, respectively. However, these figures were based on an assumption that 100% replacement by the heavy isotopes was achieved. Filner and Varner (1967), based on the amino acid composition of α-amylase, have reported that the peptide carbonyl oxygens account for 14.4% of the mass. They expected that the increase in the mass of α-amylase in the presence of 80% $H_2{}^{18}O$ would be 0.72%; however, they obtained 1.1% increase in mass. Jacobsen and Varner (1967) also found 0.76% density increase for barley aleurone protease labeled with $H_2{}^{18}O$. If we assume that xylanase has about the same number of the peptide carbonyl oxygens as α-amylase does, the same percentage increase in mass (0.72%) will be applied to xylanase as well. The possible explanations for the difference between the observed density and the expected density increase are as follows:

1. The buoyant density of a protein is increased at the high salt gradient (Ifft and Vinograd 1966).

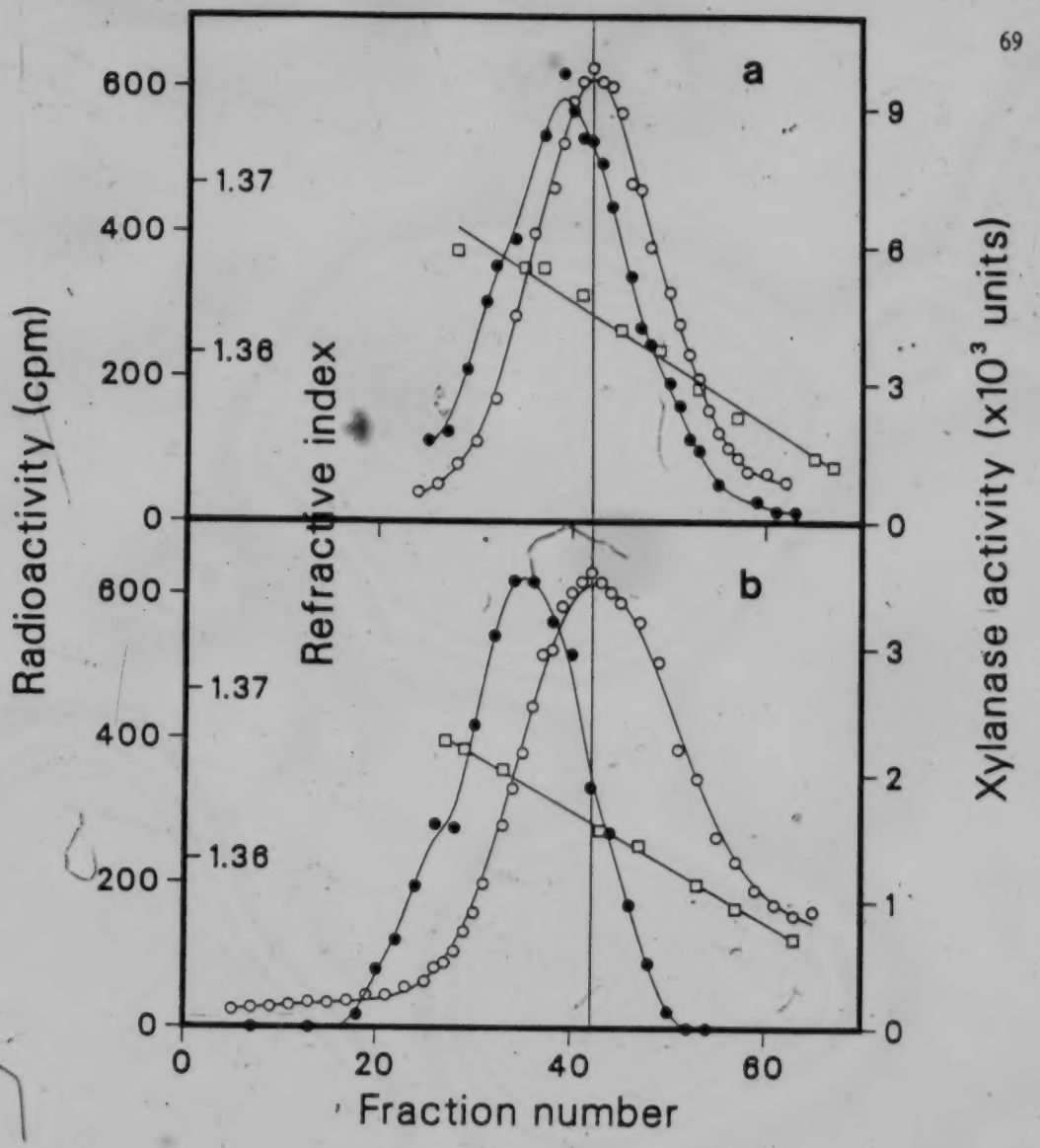



**Figure 23.** Distribution of barley aleurone xylanase in $H_2O$ and $D_2O$. Barley aleurone layers were incubated for 120 h at 25°C in 20 mM succinic acid, 10 $\mu$M $GA_3$ and 20 mM $Ca(NO_3)_2$ (pH 5.5) prepared in $H_2O$ (a) or 75% $D_2O$ (b). The enzyme was prepared from the incubation medium and the layer extract, mixed with $^{14}C$-methylated ovalbumin and CsCl, and subjected to an isopycnic equilibrium sedimentation as described in Materials and methods. ●-● Xylanase activity, o-o radioactivity, □-□ refractive index.

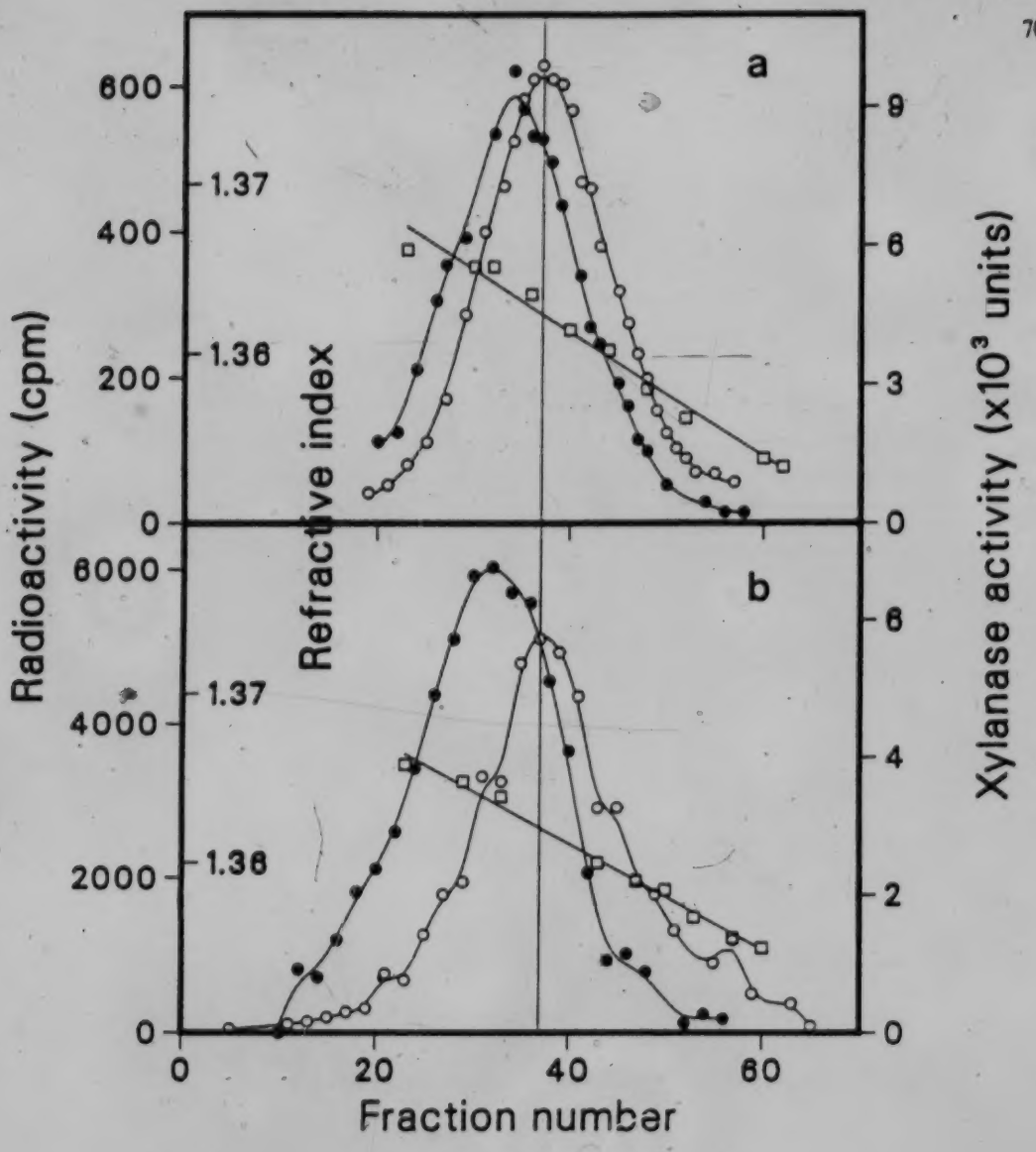



Figure 24. Distribution of barley aleurone xylanase in $H_2O$ and $H_2{}^{18}O$. Barley aleurone layers were incubated for 120 h at 25°C in 20 mM succinic acid; 10 μM and 20 mM $Ca(NO_3)_2$ (pH 5.5) prepared in $H_2O$ (a) or 80% $H_2{}^{18}O$ (b). The enzyme was prepared from the incubation medium and the layer extract, mixed with $^{14}C$-methylated ovalbumin and CsCl, and subjected to an isopycnic equilibrium sedimentation as described in Materials and methods. ●-● Xylanase, o-o radioactivity, □-□ refractive index.

**Table 5.** Densities of barley aleurone xylanase and ovalbumin marker from density labeling study. Barley aleurone layers were incubated for 120 h at 25°C in 20 mM succinic acid, 10 $\mu$M and 20 mM $Ca(NO_3)_2$ (pH 5.5) prepared in $H_2O$, $D_2O$ or $H_2{}^{18}O$. After incubation, xylanase was prepared, mixed with $^{14}C$-methylated ovalbumin and saturated CsCl, and applied to an isopycnic equilibrium sedimentation as described in Materials and methods. The values represent the mean of four experiments ±SD or two experiments ±SD for ovalbumin and xylanase in $H_2O$ or $D_2O$ and $H_2{}^{18}O$, respectively.

| Sample | Density (g/ml) | Density shift from $H_2O$ (g/ml) |
|---|---|---|
| $^{14}C$-methylated ovalbumin | 1.2942 ± 0.0112 | |
| xylanase in $H_2O$ | 1.3075 ± 0.0039 | |
| xylanase in $D_2O$ | 1.3218 ± 0.0085 | 0.0143 |
| xylanase in $H_2{}^{18}O$ | 1.3191 ± 0.0062 | 0.0116 |

2. Exchange between $H_2{}^{18}O$ and the peptide carbonyl oxygen may be higher than the percentage used for the calculation. The percentage of 0.72% was obtained when only one-half atom of oxygen was labeled (see equation 2).

3. Exchange may occur between $H_2{}^{18}O$ and oxygens other than the peptide carbonyl oxygen.

4. The newly synthesized protein may trap some of $H_2{}^{18}O$ inside the molecule as it folds.

5. The protein has a carbohydrate portion in its molecule. In other words, it is a glycoprotein. Since carbohydrate is an oxygen-rich component, the possibility of exchange between $^{18}O$ from $H_2{}^{18}O$ and $^{16}O$ in carbohydrate molecule may occur. The density of carbohydrate is also higher than that of protein. The density of carbohydrate is about 1.5-1.7 g/ml while that of protein is 1.27-1.35 g/ml (Anstine *et al.* 1970).

In addition, the last three possibilities also give a false positive for the density shift. As mentioned earlier, the shift in density of 0.045 g/ml can be expected in protein labeled with 75% $D_2O$ (Hu *et al.* 1962). Other workers, working with 75-80% $D_2O$ found that the increase in the density was 0.0163 (1.27%) and 0.0174 (1.30%) g/ml for barley half-seed $\beta$-glucanase and ribonuclease, respectively (Bennett and Chrispeels 1972), and 0.0217 (1.6%) and 0.01 g/ml for germinating barley acid phosphatase (Chrispeels and Varner 1973) and peroxidases (Anstine *et al.* 1970), respectively. The possible shift in the density due to the exchange between the deuterium of the medium and the preexisting protein, and the trapping of $D_2O$ in the $\alpha$-amylase molecule were proved to be negligible (0.0008 g/ml, Anstine *et al.* 1970). The shift in the density contributed by carbohydrate was also ruled out since xylanase does not contain a carbohydrate group (page 36).

The results of the inhibitor study and the density labeling study indicate that there is a *de novo* synthesis of barley aleurone xylanase in response to $GA_3$. In addition, when the aleurone layers were incubated with the routine incubation medium plus $^{35}S$-methionine and the xylanase prepared from the incubation medium was run on the SDS-PAGE, the

incorporation of $^{35}S$-methionine in the proteins of molecular weights of 30-35 kD were observed on a fluorogram (results not shown). A better result on the incorporation of labeled amino acid into the xylanase molecule can be obtained if the sample is electrophoresed on a non-denaturing gel system and the bands of protein obtained from a fluorogram are matched to an activity stain for xylanase. However, an activity stain for xylanase in barley aleurone system has not yet been achieved. This failure may result from the lack of a good non-denaturing system for PAGE. An activity stain for xylanase from fungi on PAGE has been reported (Biely *et al.* 1985, MacKenzie and Williams 1984).

**J. Role of calcium and calmodulin on the synthesis and release of xylanase**

In the experiments on $GA_3$-induced α-amylase in cereal aleurone layers, inclusion of $Ca^{2+}$ in the incubation medium is always required. It has been suggested that $Ca^{2+}$ is essential for the stability of α-amylase and it has been shown recently to be required for the synthesis and secretion of barley aleurone α-amylase (Chrispeels and Varner 1967, Deikman and Jones 1985). Calcium was also included in the incubation medium for the experiments on barley xylanase by previous workers (Dashek and Chrispeels 1977, Eastwell and Spencer 1982b, Taiz and Honigman 1976) but its effects on xylanase have never been studied.

*Effects of calcium and other ions on the synthesis and release of xylanase*

Table 6 shows the effects of $GA_3$, $Ca^{2+}$ and certain other ions on the synthesis and release of xylanase. $GA_3$ or $Ca^{2+}$ alone did not induce a large increase in xylanase activity. However, when $GA_3$ was applied simultaneously with $Ca^{2+}$, the synthesis increased tremendously. It is evident from Table 6 that it is $Ca^{2+}$, not the anions $Cl^-$ or $NO_3^-$, that is required for the $GA_3$-induced xylanase activity since $CaCl_2$ or $Ca(NO_3)_2$ gave similar results while $NaNO_3$ induced only a small increase. Calcium has been shown to be essential for the synthesis and secretion of α-amylase in barley and rice (Deikman and Jones 1985, Mitsui *et al.* 1984). Other ions such as the monovalent cation, $Na^+$, had only small effect on xylanase

**Table 6.** Effects of calcium and other ions on the synthesis and release of barley aleurone xylanase. Ten aleurone layers were incubated for 72 h at 25°C in each of the following media: succinate buffer or succinate buffer plus $GA_3$, $Ca(NO_3)_2$, $GA_3+Ca(NO_3)_2$, $GA_3+CaCl_2$, $GA_3+NaNO_3$, $GA_3+Mg(NO_3)_2$, or $GA_3+SrCl_2$. Details on each medium are given in Materials and methods. Xylanase in the incubation medium and in the layer extract was prepared and assayed as described in Materials and methods. The values represent the mean of at least four different determinations ±SD.

| Addition to succinate | Xylanase activity (munits/layer) | |
|---|---|---|
| | Medium | Extract |
| — | 0 ± 0 | 0.04 ± 0.05 |
| $GA_3$ | 0.10 ± 0.09 | 0.07 ± 0.03 |
| $Ca(NO_3)_2$ | 0.31 ± 0.31 | 0.24 ± 0.21 |
| $GA_3+Ca(NO_3)_2$ | 16.14 ± 4.18 | 0.89 ± 0.20 |
| $GA_3+CaCl_2$ | 15.68 ± 1.97 | 0.38 ± 0.21 |
| $GA_3+NaNO_3$ | 1.44 ± 0.41 | 0.08 ± 0.03 |
| $GA_3+Mg(NO_3)_2$ | 10.09 ± 1.77 | 0.13 ± 0.02 |
| $GA_3+SrCl_2$ | 18.26 ± 1.29 | 0.18 ± 0.03 |

synthesis (Table 6). The divalent cations $Mg^{2+}$ or $Sr^{2+}$ could replace $Ca^{2+}$ for xylanase synthesis. The total activity of xylanase produced in the presence of $Mg^{2+}$ and $Sr^{2+}$ was 60 and 108%, respectively, of that in the presence of $Ca^{2+}$. Magnesium has been found to be unable to replace $Ca^{2+}$ for α-amylase synthesis and release (Chrispeels and Varner 1967, Mitsui *et al.* 1984). Strontium substitutes for $Ca^{2+}$ in many biochemical processes. The ability of $Sr^{2+}$ to substitue for $Ca^{2+}$ has been shown in the synthesis and secretion of α-amylase in barley and rice (Jones and Jacobsen 1983, Mitsui *et al.* 1984).

As has been discussed earlier, $Ca^{2+}$ was not required for xylanase activity and $Cl^-$ inhibited xylanase (page 49). From Table 6, however, $Ca^{2+}$ was definitely essential for xylanase synthesis and $Cl^-$ did not affect the synthesis. Any effects of $Ca^{2+}$ on the release of the enzyme were not discernible since $Ca^{2+}$ also affects the synthesis. It has been reported that the omission of $Ca^{2+}$ from the incubation medium suppresses the release of α-amylase, acid phosphatase, β-glucanase, protease and ribonuclease from barley aleurone layers (Jones and Jacobsen 1983). It is noteworthy that the requirement of $Ca^{2+}$ for the release of those enzymes may partly result from the induction by $Ca^{2+}$ of synthesis of xylanase, which in turn hydrolyzes the cell walls.

*Effects of different calcium concentrations on the synthesis and release of xylanase*

The effects of various concentrations of $Ca^{2+}$, supplied as $Ca(NO_3)_2$, on the levels of xylanase in the presence of $GA_3$ are shown in Figure 25. In the presence of 1 mM $Ca^{2+}$, xylanase activity was at least 60 times higher than that in its absence. Maximum xylanase activity was obtained at $Ca^{2+}$ concentrations from 5-40 mM. This figure was similar to the $Ca^{2+}$ concentration required for barley α-amylase (5-20 mM, Chrispeels and Varner 1967, Jones and Carbonell 1984) but was much higher than that reported for rice α-amylase (0.5 mM, Mitsui *et al.* 1984).

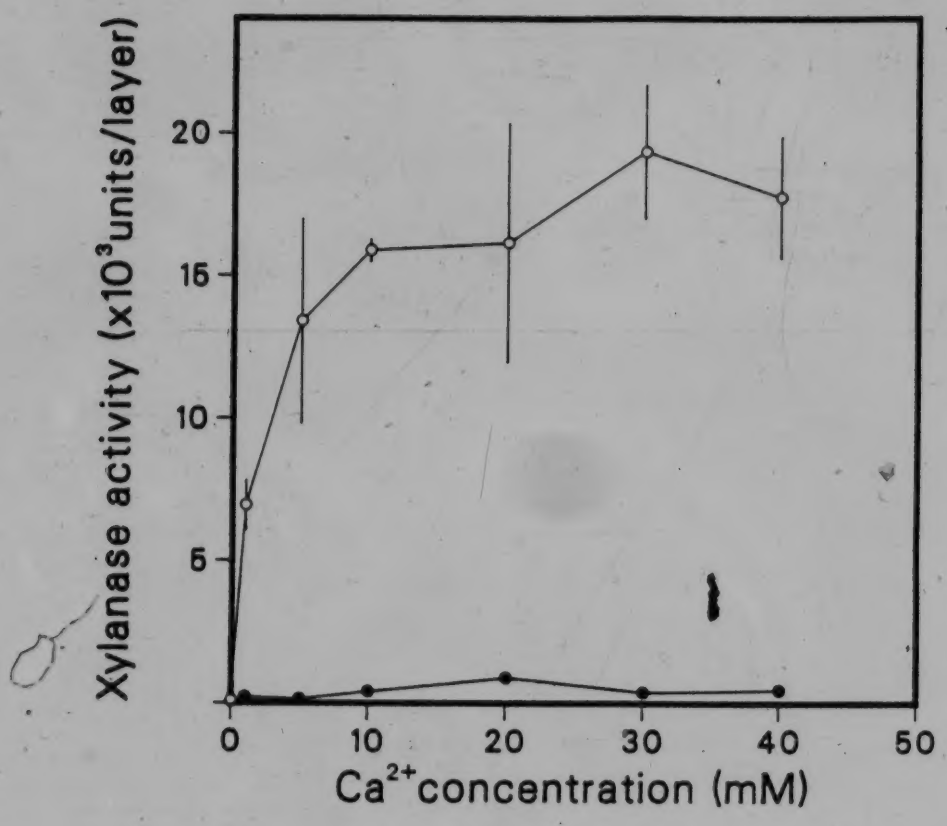



**Figure 25.** Effects of calcium concentration on the synthesis and release of barley aleurone xylanase. Ten aleurone layers were incubated for 72 h at 25°C in various concentrations of $Ca(NO_3)_2$ prepared in 10 $\mu$M $GA_3$ and 20 mM succinic acid (pH 5.5). Xylanase in the incubation medium (o-o) and layer extract (●-●) was prepared and assayed as described in Materials and methods. Bars represent ±SD.

*Effects of calmodulin and calmodulin antagonists on the synthesis and release of xylanase*

Since $Ca^{2+}$ was shown to be involved in the regulation of the synthesis of xylanase (Table 6 and Figure 25), calmodulin (CaM), which is a ubiquitous and highly-conserved $Ca^{2+}$-binding protein, may act as a signal transducer of $Ca^{2+}$. Calmodulin exhibits $Ca^{2+}$-dependent regulatory activities toward several enzymes and proteins (Cormier 1983). Calmodulin has been found in plants, including barley (Grand *et al.* 1980, Marmé and Dieter 1983). It binds to phenothiazine drugs, e.g. chlorpromazine (CPZ) and trifluoperazine (TFP) in a $Ca^{2+}$-dependent manner.

Table 7 shows the effects of CaM as well as its antagonists, CPZ and TFP, on the synthesis and release of xylanase. In the presence of $GA_3$ and $Ca^{2+}$, calmodulin at either 0.07 or 5 μg/ml did not significantly improve xylanase activity over that obtained with $GA_3$ and $Ca^{2+}$ alone. Calmodulin accompanied by $GA_3$, in the absence of $Ca^{2+}$, caused a small increase in the levels of xylanase. Small change in activity was also obtained when 30 μM CPZ or TFP was added to the $GA_3$ and $Ca(NO_3)_2$ medium. Chlorpromazine and trifluoperazine at 500 μM decreased the synthesis as well as the release of xylanase. The effect of TFP was stronger than that of CPZ, at the same concentration. When 0.07 μg/ml of CaM was applied simultaneously with its antagonists, the inhibition on both synthesis and release of xylanase was lessened. The total activity of xylanase obtained in the presence of CaM+CPZ and CaM+TFP was 73 and 35%, respectively, of that in the absence of the antagonists. Full activity was not regained. This may result from an inadequate amount of CaM and/or the non-specific effects of the phenothiazine drugs on the aleurone cells as have been found in animal cells (Roufogalis 1982, Roufogalis 1985). Calmodulin cannot completely reverse the inhibition of rat erythrocyte $Ca^{2+}$-$Mg^{2+}$ ATPase by 100 μM TFP (Levin and Weiss 1980). Although CPZ and TFP were applied at commonly used concentrations, those that inhibited the synthesis of xylanase are probably too high to specifically demonstrate the role of CaM in the synthesis of xylanase. Roufogalis (1982) has recommended the use of concentrations of 10-30 μM of phenothiazine drugs for CaM antagonism, since these drugs are not specific for CaM. They are found to

**Table 7.** Effects of calmodulin, chlorpromazine and trifluoperazine on the synthesis and release of barley aleurone xylanase. Ten aleurone layers were incubated for 72 h at 25°C in the incubation medium shown below. Each medium was prepared in succinate buffer (pH 5.5) and the concentration of each chemical was: 20 mM succinic acid, 10 μM $GA_3$, 20 mM $Ca(NO_3)_2$, 0.07 or 5 μg/ml CaM, 30 or 500 μM CPZ and 30 or 500 μM TFP. Xylanase in the incubation medium and in the layer extract was prepared and assayed as described in Materials and methods. The values represent the mean of at least four different determinations ±SD.

| Incubation medium | Xylanase activity (munits/layer) | |
|---|---|---|
| | Medium | Extract |
| succinate | 0 ± 0 | 0.04 ± 0.05 |
| $GA_3$ | 0.10 ± 0.09 | 0.07 ± 0.03 |
| $Ca(NO_3)_2$ | 0.31 ± 0.31 | 0.24 ± 0.21 |
| 0.07 μg/ml CaM | 0.27 ± 0.05 | 0.23 ± 0.10 |
| $GA_3$+$Ca(NO_3)_2$ | 16.14 ± 4.18 | 0.89 ± 0.20 |
| $GA_3$+0.07 μg/ml CaM | 0.68 ± 0.20 | 0.12 ± 0.01 |
| $GA_3$+$Ca(NO_3)_2$+0.07 μg/ml CaM | 17.27 ± 2.84 | 0.36 ± 0.07 |
| $GA_3$+$Ca(NO_3)_2$+5 μg/ml CaM | 18.51 ± 2.52 | 0.55 ± 0.10 |
| $GA_3$+$Ca(NO_3)_2$+30 μM CPZ | 15.94 ± 1.01 | 1.19 ± 0.15 |
| $GA_3$+$Ca(NO_3)_2$+500 μM CPZ | 7.88 ± 1.38 | 1.34 ± 0.32 |
| $GA_3$+$Ca(NO_3)_2$+500 μM CPZ+0.07 μg/ml CaM | 11.90 ± 1.79 | 0.93 ± 0.24 |
| $GA_3$+$Ca(NO_3)_2$+30 μM TFP | 17.22 ± 1.12 | 1.20 ± 0.22 |
| $GA_3$+$Ca(NO_3)_2$+500 μM TFP | 0.24 ± 0.21 | 1.88 ± 0.59 |
| $GA_3$+$Ca(NO_3)_2$+500 μM TFP+0.07 μg/ml CaM | 1.32 ± 0.36 | 4.94 ± 0.73 |

bind to other $Ca^{2+}$-binding proteins such as troponin C (Levin and Weiss 1978). The requirement of higher concentration of drugs in the present study may partly result from a low uptake of the drugs by aleurone tissues. Our studies on the possible role of CaM in the synthesis of xylanase are only preliminary and they require much more work before they can be interpreted with any degree of confidence. However, CaM is one of the possible factors that may be involved in $Ca^{2+}$-induced xylanase synthesis and release.

Calcium has been reported to control many physiological processes in plants, e.g. cell elongation, cell division, enzyme secretion and hormone action (Hanson 1984, Hepler and Wayne 1985). Calcium has been categorized as a second messenger in animal cells for years but this role has been accepted only recently for plant cells. The other common second messenger in animal cells is cAMP. Although cAMP is present in some plant cells, its role as a second messenger has never been proved. It is not known whether cAMP is present in aleurone cells.

The results shown above indicated that $Ca^{2+}$ regulates the synthesis and release of xylanase but the mechanism is unknown. The general mechanism by which $Ca^{2+}$ modulates a response is through a change in its free concentration within the cytosol (Hepler and Wayne 1985). At a high concentration, $Ca^{2+}$ can bind either directly to a target protein and get the response or to a modulator such as CaM. The latter may be possible in the regulation of xylanase synthesis since CaM and its antagonists changed the synthesis and release of xylanase (Table 7). The $Ca^{2+}$-CaM complex may play a role in the activation of some proteins that then activate the synthesis and release of xylanase. Calcium-dependent protein kinase and protein phosphorylation have been found recently in pea shoot (Hetherington and Trewavas 1982, Marmé and Dieter 1983). Calcium ions were suggested to be required for the translation of α-amylase mRNA or for the posttranslational processing or transport of group B-α-amylase, not at the transcriptional level (Deikman and Jones 1985, Deikman and Jones 1986).

As has been dicussed earlier, the free $Ca^{2+}$ concentration in the cytosol controls many processes. However, little is known about $Ca^{2+}$ concentration in aleurone cells or about the transportation of $Ca^{2+}$ from the incubation medium to the cells. Two mechanisms of $Ca^{2+}$ transport have been described. One mechanism is $Mg^{2+}$-requiring, CaM-stimulated $Ca^{2+}$-ATPase and the other is $Ca^{2+}/H^{+}$ exchange that is dependent on a proton motive force (Hanson 1984, Zucchi and Hanson 1983). The latter mechanism may act in the transport of $Ca^{2+}$ into aleurone cells since a drop in the pH of incubation medium was observed (page 39). Passive transport of $Ca^{2+}$ may also be involved in $Ca^{2+}$ transport into aleurone cells, as has been suggested for the transport of $Ca^{2+}$ in roots in two legumes and three cereals (Hanson 1984, Monestiez *et al.* 1982). Hamabata *et al.* (1986) have proposed that the source of $Ca^{2+}$ in a germinating wheat is the endosperm, in which the $Ca^{2+}$ is solubilized by the acidity produced by aleurone cells. However, more investigations are needed in support their proposal.

## V. GENERAL DISCUSSION AND CONCLUSIONS

The discovery of arabinoxylan as a major component of barley aleurone cell wall has interested researchers who work in the field of seed germination and nutrient mobilization in an enzyme, xylanase, that hydrolyzes the xylan backbone of the arabinoxylan. A possible role of this enzyme is that it may be involved in the cell wall degradation of aleurone cells and permit the release of other hydrolytic enzymes from aleurone to endosperm. However, supporting evidence has been lacking.

In the present research, one and the major, form of xylanase was purified from barley aleurone layers. The purification procedure was chosen so that the enzyme could be obtained in a few steps. The enzyme was purified 29 fold. The enzyme was relatively small in size. The molecular weights were found to be 34 or 27 kD, as determined by SDS-PAGE or Bio-Gel P-200 gel filtration chromatography, respectively. It gave a negative stain with a PAS reagent for a glycoprotein. The pI of xylanase was 4.6. The optimum pH was 5.5 for both activity and stability. It has been mentioned in a review by Fry (1984) that cells can control the activities of wall enzymes by regulating the pH within the walls. Cells can manipulate pHs between 4 and 6.

The optimum temperatures for the activity and for stability of xylanase were 35 and 0-4°C, respectively. The Km and Vmax were 0.86 mg of xylan/ml and 0.014 units, respectively. Bromate, $Hg^{2+}$, $Cu^{2+}$, $Zn^{2+}$ and SDS strongly inhibited xylanase activity. Chloride ions and EGTA inhibited xylanase at 40 and 20 mM, respectively. Calcium ions, $Na^{+}$, $NO_3^{-}$ and $SO_4^{2-}$ had no effect on xylanase.

The simultaneous development of xylanase in the aleurone layers and appearance of α-amylase, $K^{+}$, $Mg^{2+}$ and $PO_4^{3-}$ in the incubation medium (Figures 19 and 20), in the presence of $GA_3$, and the demonstration of aleurone cell walls hydrolysis by a purified xylanase (Figures 21 and 22) suggest a role for xylanase in the release of α-amylase and ions from the aleurone cells to the endosperm. These results support a proposal of Varner and Mense (1972), and Ashford and Jacobsen (1974) that the release of α-amylase and other hydrolases

is limited by cell walls. In future work, further supporting evidence may be obtained by incubating aleurone layers with a purified aleurone xylanase in the absence of $GA_3$ and demonstrating the appearance of hydrolytic enzymes such as acid phosphatase or $\beta$-glucanase in which the synthesis does not depend on $GA_3$.

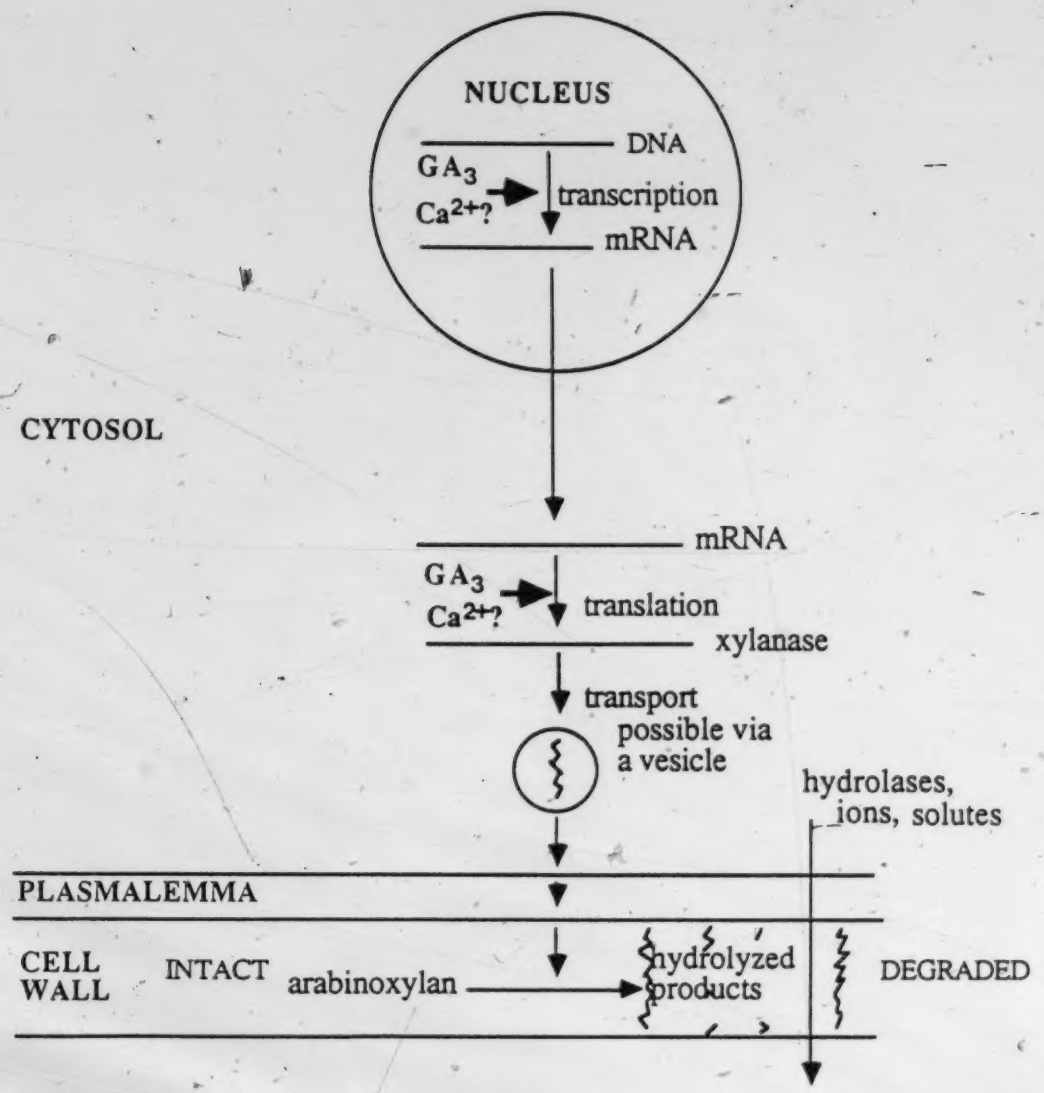
The induction of xylanase by $GA_3$ and $Ca^{2+}$ has been demonstrated (Figures 18, 25 and Table 6). The enhancement of xylanase levels by $GA_3$ and $Ca^{2+}$ is inhibited by cycloheximide and cordycepin (Table 4). The shift in the density of xylanase in the presence of $D_2O$ or $H_2{}^{18}O$ (Figures 23, 24 and Table 5) implies that the enzyme incorporates the heavy isotope into its molecule. These results suggest that $GA_3$ and $Ca^{2+}$ induce the *de novo* synthesis of xylanase as has been reported for other hydrolytic enzymes in the same system (Filner and Varner 1967, Jacobsen and Varner 1967).

The mechanism of how $GA_3$ and $Ca^{2+}$ modulate the synthesis of xylanase has not yet been studied. The effects of $Ca^{2+}$ on $\alpha$-amylase synthesis (Deikman and Jones 1985, Deikman and Jones 1986) indicate that $GA_3$ increases $\alpha$-amylase mRNA while $Ca^{2+}$ affects the translation or posttranslation of $\alpha$-amylase. Our results with cycloheximide and cordycepin inhibition suggest that $GA_3$ and $Ca^{2+}$ may regulate xylanase at transcription and/or translation. The role of $GA_3$ and $Ca^{2+}$ on the release of xylanase is not so clear since the synthesis and the release are very closely related. Nevertheless, the results of experiments with phenothiazine drugs and CaM (Table 7) showed that CaM may be involved in the $Ca^{2+}$-induced xylanase synthesis. In addition, the effect of $Ca^{2+}$ on the release of xylanase may be increased by use of those inhibitors.

The overall results from this research and the knowledge from $\alpha$-amylase research permit the proposal of the following model (Figure 26) for the control of xylanase synthesis and the role of xylanase in barley aleurone cells. $GA_3$ and $Ca^{2+}$ control the synthesis of xylanase at the transcription of xylanase mRNA and/or translation of xylanase. After xylanase is synthesized, it is transported, possibly via a secreted vesicle, to the plasmalemma (Chrispeels 1976) and secreted to the cell wall where it hydrolyzes the arabinoxylan within the

**Figure 26.** Diagrammatic representation of the control of the synthesis of xylanase and the role of xylanase in cell wall degradation and release of enzymes in barley aleurone cells. GA, and $Ca^{2+}$ regulate the transcription and/or translation steps for the synthesis of xylanase. The enzyme is transported, possibly via a vesicle to the plasmalemma and secreted to hydrolyze arabinoxylan in the cell wall. The degraded wall facilitates the release of hydrolases, ions and other solutes from aleurone cell to outside.

## ALEURONE CELL



wall to smaller compounds. The intact cell wall becomes degraded and then permits the release of hydrolases, ions and other solutes from the aleurone cell to the outside.

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